Angiogenesis and vascular leakage in diabetic retinopathy

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Doctor of Philosophy in Cell Biology

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Declaration

I, William Edward Laughlin can confirm that work presented in this thesis is my own and where information has been derived from other source this has been appropriately referenced and acknowledged.

Abstract

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and a leading cause of blindness. Increased vascular permeability in the retina following blood-retinal barrier (BRB) breakdown is a clinically significant event and a major cause of vision loss. VEGF blockade, despite being the only treatment to improve visual acuity, has a limited effectiveness for a majority of patients. A significant proportion of patients develop resistance to treatment, which implies that other factors are also involved in the pathology of this disease. There is currently a major unmet clinical need for therapeutics which target the early stages of DR prior to the onset of overt vascular symptoms.

The aim of this thesis was to investigate early diabetes-induced changes to the retina and their effects on the vasculature, in order to identify novel potential therapeutic targets. This was achieved by investigating the effects of high glucose and glycated albumin on the vasculature using the mouse metatarsal assay, an *ex vivo* model of angiogenesis and the effects of diabetes on the retina with the streptozotocin-induced diabetic mouse.

Both high glucose and glycated albumin altered angiogenesis in the metatarsal assay. Investigation of the diabetic mouse retina revealed evidence of increased inflammation and oxidative stress at the cellular and molecular level, accompanied with evidence of vascular leakage. qPCR analysis revealed an increase in *Angptl6* and *Lrg1* expression of which had not been investigated in the diabetic mouse retina before. Studies with transgenic mouse models implied that Lrg1 is involved in the early stages of pathophysiology of DR and may be a suitable therapeutic target prior to the onset of overt vascular symptoms.

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List of Abbreviations

4-HNE - 4-Hydroxynonenal

AGE - advanced glycation endproduct

AGE-BSA - Advanced glycation end product bovine serum albumin

Alk - activin receptor-like kinase

AMD - age related macular degeneration

Ang - angiopoietin

Angptl-4 - angiopoietin like 4

AP-1 - activator protein 1

BCL-2 - B-cell lymphoma 2

Bis-Tris - 2,2-Bis(hydroxymethyl)-2,2’,2”-nitrilotriethanol, 2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol, Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane

BRB - blood retinal barrier

BSA - Bovine serum albumin

CD - cluster of differentiation

cDNA - complementary DNA

CNV - choroidal neovascularisation

Cy3 - cyanine 3

DAPI - 4',6-diamidino-2-phenylindole

Dll4 - Delta-like 4

DMO - diabetic macular oedema

DR - diabetic retinopathy

DTT - dithiothreitol

ECM - extracellular matrix

ELISA - enzyme linked immunosorbent assay

eNOS - endothelial nitric oxide synthase

EPC - endothelial progenitor cell

ESAM - endothelial-cell specific adhesion molecule

FA - fluorescein angiography

FBS - fetal bovine serum

FGF-2 - fibroblast growth factor 2

FITC - fluorescein isothiocyanate

Foxo - forkhead box

GFAP - glial fibrillary acidic protein

GLUT-1 - glucose transporter 1

GLUT-2 - glucose transporter 2

HIF-1α - hypoxia inducible factor 1α

HRE - hypoxia response element

HSPG - heparan sulphate proteoglycan

HUVEC - human umbelical vein endothelial cells

i.p. - intraperitoneal

Iba1 - ionised calcium-binding adapter molecule 1

ICAM-1 - intercellular adhesion molecule 1

IF - immunofluorescence

IgG - immunoglobulin G

IHC - immunohistochemistry

IL - interleukin

IVC - individually ventilated cage

Jag-1 - jagged 1

JAK/STAT -janus kinase/signal transducer and activator of transcription

JAM - junctional adhesion molecule

KI - knock in

KO - knock out

LRG1 - leucine rich glycoprotein 1

MAPK - mitogen activated protein kinase

MCP-1 - macrophage chemoattractant protein 1

MFSD2A - major facilitator superfamily domain containing 2A

MMP - matrix metalloproteinase

mRNA - messenger RNA

N-cadherin - neural cadherin

NADPH - nicotinamide adenine dinucleotide phosphate

Nrp-1 – neuropilin 1

NF-κB - nuclear factor κ-light-chain-enhancer of activated B cells

NG-2 - neural/glial antigen 2

NPDR - nonproliferative diabetic retinopathy

Nrp - neuropilin

O-GlcNAc - O-Linked β-N-Acetylglucosamine

OCT - optimal cutting temperature compound

OIR - oxygen induced retinopathy

p-value - probability value

PARP - Poly-ADP ribose polymerase

PBS – phosphate buffered saline

PDGF - platlet derived growth factor

PDGFR - platlet derived growth factor receptor

PDR - proliferative diabetic retinopathy

PFA - paraformaldehyde

PLVAP - plasmalemma vesicle-associated protein

qPCR - quantitative polymerase chain reaction

RAGE - receptor for advanced glycation end products

ROS - reactive oxygen species

SD - standard deviation

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM - standard error of the mean

SMA - smooth muscle actin

Smad - small mothers against decapentaplegic

Sp-1 – Specificity protein 1

STAT3 - signal tranducer and activator of transcription 3

TBST - tris-buffered saline with 0.1% Tween-20

TGF - transforming growth factor

TGFβR - transforming growth factor β receptor

Tie - tyrosine kinase with immunoglobulin-like and EGF-life domains

TNF-α - tumour necrosis factor α

VCAM-1 - vascular cell adhesion molecule 1

VE-cadherin - vascular endothelial cadherin

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

VVO - vesiculo-vacuolar organelle

WT - wild type

ZO-1 - zonula occludens 1

Chapter 1

Introduction

1 Vascular endothelium and angiogenesis

Angiogenesis is the growth and formation of new blood vessels, which is essential for development of complex organisms (Ribatti & Crivellato 2012). In addition to its essential physiological role, it also contributes to the pathophysiology of various disease (Carmeliet 2003). Insufficient angiogenesis contributes to stroke, myocardial infarction and neurodegeneration, whilst excessive angiogenesis supports the growth of solid tumours, contributes to artherosclerosis and causes retinal damage in age related macular degeneration (AMD) and diabetic retinopathy (DR) (Carmeliet 2003).

In mature healthy tissue, the vascular endothelium is predominantly in a quiescent state. Endothelial cells form a monolayer with varying degrees of pericyte coverage and associations with other perivascular cells depending on the vascular bed (Armulik et al. 2011). The vascular endothelium has multiple mechanisms that act in concert to maintain vascular integrity and quiescence. These include; autocrine and paracrine signalling (Cleaver & Melton 2003), intercellular junctional complexes (Dejana 2004) and interactions with the extracellular matrix (ECM) mediated by integrin signalling (Jain 2003). One unifying concept to both pathological vascular dysfunction and developmental angiogenesis is that multiple pro-angiogenic signals must converge to overcome these different regulatory mechanisms to induce a phenotypic switch from a quiescent to activated vessel.

1.1 Stimulation of angiogenesis

Angiogenesis can be stimulated by a variety of signals induced by various stimuli such as hypoxia and inflammation (Pugh & Ratcliffe 2003; Kim et al. 2013). These activate a sequence of events that permit the sprouting of new vessels. Vascular endothelial growth factor (VEGF) is the most well known inducer of angiogenesis. Regulation of VEGF transcription by hypoxia is regulated by hypoxia response elements (HRE) which are binding sites for the oxygen sensing transcription factor hypoxia inducible factor 1 α (HIF-1α) (Pugh & Ratcliffe 2003; Vinores et al. 2006). Additionally VEGF mRNA stability is increased in hypoxia (Claffey et al. 1998). VEGF is also induced by the pro-inflammatory cytokines interleukin (IL)-1β (Ben-Av et al. 1995) and IL-6 (Cohen et al. 1996).

There are many other factors involved in the induction of angiogenesis (Ferrara 2010), however, VEGF is considered to be one of the most important growth factors in both physiological and pathological angiogenesis. VEGF promotes proliferation, survival and migration of endothelial cells, predominantly through VEGFR2 signalling (Ferrara 2001). Transforming growth factor β (TGF-β) is another growth factor, which induces or inhibits angiogenesis in a context dependent manner. Signalling through transforming growth factor β receptor 2 (TGFβR2), activin receptor-like kinase 1 (Alk1) and endoglin promotes angiogenesis through small mothers against decapentaplegic (Smad) 1/5/8 activation, whilst signalling through TGFβR2 and activin receptor-like kinase 5 (Alk5) promotes vessel quiescence through Smad 2/3 activation (Pardali et al. 2010). The regulation of the context dependent activity of TGF-β signalling in

endothelial cells therefore plays a key role in the regulation of angiogenesis. This means the vascular microenvironment can determine the pro/anti-angiogenic state of the vasculature and highlights the complex and multiple steps that regulate angiogenesis.

For angiogenesis to occur, space must be created through remodelling of the basement membrane, which is mediated by matrix metalloproteinase (MMP) proteolysis. This releases angiogenic factors stored within the matrix (Bergers et al., 2000) and activates growth factors through proteolytic cleavage (Iruela-Arispe & Davis, 2003). Vascular permeability is increased due to vascular endothelial-cadherin (VE-cadherin) and other junctional proteins become phosphorylated and internalised (Dejana 2004), which weakens junctions between endothelial cells. Increased permeability allows the release of plasma proteins, which deposits new extracellular matrix (ECM) proteins and release of angiogenic factors (Carmeliet 2000). The newly formed ECM provides a scaffold and a gradient of signals to guide endothelial cell migration (Carmeliet & Jain 2011). The ECM scaffold plays an active and important role in regulating growth factor signalling to migrating cells. For example, deleting the heparan sulphate proteoglycan (HSPG) retention motif in platelet derived growth factor B (PDGF-B) impairs pericyte recruitment (Lindblom et al. 2003).

Disrupting the VE-cadherin junctions also sensitises endothelial cells to angiogenic stimuli by releasing growth factor receptors such as VEGFR2, and transcription factors such as β-catenin which are tethered in an inactive conformation (Figure 2) (Giannotta et al. 2013). This shows that pro-

angiogenic signalling pathways can self-regulate through a positive feedback mechanism, which sensitises target endothelial cells and self-amplify their own signalling pathways.

Angiopoietin-2 (Ang-2) is produced in endothelial cells and promotes vessel remodelling, vascular permeability and angiogenesis in both physiological and pathological angiogenesis (Felcht et al. 2012). Ang-2 is rapidly released from intracellular organelles known as Weibel-Palade bodies in response to vascular permeability inducing factors (Fiedler et al. 2004). Storage of Ang-2 and other factors within these organelles suggests that it enables endothelial cells to rapidly respond to permeability, inflammatory and thrombotic inducing agents (Metcalf et al. 2008). Ang-2 inhibits tyrosine kinase with immunoglobulin-like and EGF-life domains (Tie)2 signalling by competing with Ang-1 (Maisonpierre et al. 1997). This leads to activation of Foxo1 transcriptional activity which promotes vessel destabilisation and remodelling through upregulation of Ang-2 and MMPs (Kim et al. 2016).

ECM remodelling, disruption of intercellular junctions and release of growth factors leads to the disruption of vascular homeostasis, and permits neovascularisation. The formation of new sprouting vessels is regulated by the selection of tip and stalk cells (Ribatti & Crivellato 2012). Tip cells have a polarized and motile phenotype, and migrate in the direction of angiogenic signals. Conversely, stalk cells have a high proliferative potential and are responsible for the *de-novo* formation of the vascular lumen, junction formation and basement membrane deposition. Selection of tip/stalk cell phenotype is determined at least in part by Notch signalling, which is

induced in stalk cells in response to increased VEGFR2 activation in tip cells (Phng & Gerhardt 2009). Acquisition of a tip or stalk cell phenotype determines how endothelial cells respond to various growth factors such as VEGF and TGF-β. Neuropilin-1 (Nrp1) is predominantly expressed in tip cells (Phng & Gerhardt 2009), where it attenuates the anti-angiogenic effect of TGF-β signalling by inhibiting Smad2/3 leading to increased pro-angiogenic signalling via Smad1/5/8 (Aspalter et al. 2015).

Whilst sprouting angiogenesis is the most widely studied mechanism of angiogenesis, other physiological and pathological mechanisms exist. In physiological conditions, circulating endothelial progenitor cells (EPCs) can be recruited to differentiate into mature endothelial cells by vasculogenesis (Calzi et al. 2010), and existing vessels can be split to form multiple new vessels by intussusception (Gianni-Barrera et al. 2013). Whilst most neovascularisation results from locally proliferating endothelial cells through sprouting angiogenesis and intussusception, the contribution of EPCs should not be discounted since they are involved in both physiological and pathological angiogenesis (Calzi et al. 2010).

1.2 Vascular stabilisation and maturation

Generating stable and perfused vessels requires the recruitment of perivascular cells and the formation of endothelial cell junctions. The termination of vascular leakage enables the vessel to function and for perfusion to take place (Jain 2003). One area where pathological and physiological angiogenesis differ is during the stabilisation and maturation stages. Both of these play a crucial role in maintaining an intact and fully

functioning vascular network. This section will focus on the context of physiological angiogenesis for the purpose of discussing vascular stabilisation and maturation.

1.2.1 Lumen formation

Formation of the lumen is essential for allowing the newly formed vessel to perfuse the newly vascularized tissue, and occurs simultaneously with sprouting. Before lumen formation can take place VE-cadherin must form junctions between endothelial cells and provide polarity to the cytoskeleton which in turn provides organisation for lumen formation (Ribatti & Crivellato 2012). Once formed, lumen diameter is dynamically regulated by; VEGF, endothelial-nitric oxide synthase (eNOS) and mural cells (Iruela-Arispe & Davis 2009) and is capable of determining blood pressure.

1.2.2 Pericyte recruitment

PDGF-B is secreted by endothelial cells undergoing angiogenesis and recruits PDGFRβ+ mural cells to the actively growing vessel (Hellström et al. 1999). PDGF-B appears to be predominantly expressed within the immature, leading edge of the vessel (Hellström et al. 1999), possibly confined to the tip cell (Armulik et al. 2011). Following PDGF-B/PDGFRβ mediated mural cell recruitment, endothelial cell derived TGF-β induces mural cell differentiation into pericytes or smooth muscle cells (Goumans et al. 2009).

Mature pericytes then secrete Ang-1, which promotes endothelial cell stability by activating Tie2 signalling in endothelial cells (Armulik et al.

2011). This promotes vascular quiescence in the newly formed vessel and leads to the resolution of the active vascular phenotype induced in response to Ang-2 signalling. The Ang-1/Tie2 signalling pathway promotes vascular homeostasis through Foxo1 phosphorylation (Daly et al. 2004), VE-cadherin (Lee et al. 2011) and occludin (Siddiqui et al. 2015) dephosphorylation and protection against advanced glycation endproduct (AGE) induced inflammatory injury (J. Zhao et al. 2015).

1.2.3 Forming the mature vascular network

A key stage in the maturation of a newly formed vascular network is for primitive vascular tubes formed during sprouting angiogenesis to undergo specialisation into arteries, capillaries and veins (Potente & Mäkinen 2017). Additional remodelling of the vascular plexus occurs through intussuception, coalescence, pruning, regression and alterations to lumen thickness to provide sufficient perfusion of the newly vascularised tissue (Ribatti & Crivellato 2012). The maturation of the vascular plexus is an essential step in physiological angiogenesis, and its absence is a hallmark of pathological angiogenesis (Jain 2003).

1.3 Vascular Permeability

The structure and function of the vascular endothelium varies according to the vascular bed, the type of vessel and temporal variations in physiological and pathological conditions. Permeability of the vascular endothelium is essential to its physiological function, enabling it to deliver nutrients and remove waste products and to permit the passage of larger objects such as cells, proteins and other larger molecules as required. During physiological

increases in permeability, elevated vascular permeability is limited and typically self-resolves (Nagy et al. 2008). This increase in permeability can be achieved through vesicular and vesiculo-vacuolar organelle (VVO), which are specialised organelles within certain endothelial cells which mediate transendothelial transport and the temporary dissolution of endothelial junctions, which permit transcellular and paracellular transport respectively (Claesson-Welsh 2015).

Whilst the permeability of endothelial cells can be dynamically regulated according to the prevailing physiological and pathological state, the heterogeneity of the vascular endothelium determines basal levels of permeability. This is achieved through the differential expression of various organelles, such as caveolae and VVOs which regulate transcellular permeability and tight junctions which control paracellular permeability (Aird 2007). In the liver the endothelium is discontinuous and fenestrated which allows efficient transfer of large molecules, whilst the vascular endothelium in the brain is continuous with very low levels of caveolae and the presence of tight junctions between endothelial cells which form the highly selective blood-brain barrier (Potente & Mäkinen 2017). These structural differences between one of the most permeable vascular beds in the body and the most carefully regulated highlights how the endothelium becomes specialised according to the physiological requirements of different tissues. Therefore when investigating diseases that affect the vasculature it is important to consider the nature of the affected vascular network when designing experiments.

1.4 Vascular Endothelium in Pathology

Whilst angiogenesis plays a key role in both physiology and development, it also plays a role in a variety of diseases such as cancer, obesity, ischemic retinopathies such as DR, and arthritis (Carmeliet 2003). Both pathological and physiological angiogenesis share many of the same mechanisms for the induction of angiogenesis; however, differences arise in their regulatory aspects and the lack of vascular maturation.

Sustained angiogenesis and vascular permeability are often mediated through chronic inflammation as observed in diabetic retinopathy (Cheung et al. 2010), or through aberrant gene expression in cancer (Welti et al. 2013). This leads to persistent pro-angiogenic/vascular permeability signalling. The result, is a vascular network which has expanded beyond what is required for tissue vascularisation, lacks appropriate mural cell coverage, has reduced basement membrane deposition, is highly permeable and is characterised by a low flow rate (Nagy et al. 2008).

VEGF plays an important role in physiological angiogenesis, its role in pathological angiogenesis in solid tumours, AMD and DR has been well established (Nagy et al. 2007). The retina is one example of a tissue where VEGF plays a fundamental role in both physiology and pathology. VEGF is produced by multiple cell types, and is essential for development, homeostasis and visual function (Sene et al. 2015). In disease, VEGF is induced by hypoxia, predominantly through transcriptional activity of HIF-1α in addition to pro-inflammatory signalling induced by the innate and adaptive immune system (Sene et al. 2015). There is extensive

heterogeneity in macrophage phenotype, where the phenotype of the macrophage population varies during different stages of angiogenesis and the presence of various cytokines in the local microenvironment (Corliss et al. 2016). This affects the level of angiogenesis and vascular remodelling by altering the *secretome* of the local macrophage population, with varying levels of VEGF, MMPs, PDGF and other growth factors depending on the macrophage population (Spiller et al. 2014; Corliss et al. 2016). The adaptive immune system can also enhance angiogenesis (Sene et al. 2015), recruited T cells secrete IL-17 which enhances the activity of VEGF and other growth factors (Takahashi et al. 2005).

Increased VEGF production during physiological conditions such as wound healing is followed by a resolution of the hypoxic or inflammatory stimuli after the tissue has been appropriately vascularised. Sustained hypoxia and inflammation in DR leads to the upregulation of VEGF, which promotes inflammation, vascular permeability and eventually angiogenesis (Penn et al. 2008). In the retina, neuronal cells express high levels of VEGFR2, which negatively regulates angiogenesis by sequestering VEGF (Okabe et al. 2014). Since neuronal cell loss has been previously reported in diabetic mice (Martin et al. 2004), this may contribute to the increased bioavailability of VEGF in the diabetic retina resulting in increased activation of VEGF signalling in the retinal vasculature. Whilst this theory has not been proven, creation of an inducible, cell specific *Vegfr2* KO mouse would allow expression of *Vegfr2* to be switched off in an adult mouse, thus avoiding issues arising during development. If VEGFR2 were to play a role in

reducing the availability of VEGF, in mice where *Vegfr2* was conditionally deleted, more free Vegf would be present in the retina.

During sustained inflammation, modulation of VEGF induced angiogenesis by inflammatory mediators is likely to alter the biology of endothelial cells resulting in pathological angiogenesis and vascular permeability. Therefore whilst anti-VEGF therapeutics are widely used in the clinic for the management of pathological angiogenesis, the role of other mediators may explain the limited effectiveness of VEGF blockade in DR and other vascular diseases, due to the activity of other compensatory pathways.

Foxo1 has been implicated in both physiological (Daly et al. 2004) and pathological (Behl et al. 2009) angiogenesis. IL-6 activates signal transducer and activator of transcription 3 (STAT3) in endothelial cells and promotes poorly regulated and pericyte deficient neovessels (Gopinathan et al. 2015). Whilst this has not been shown in endothelial cells, pSTAT3 enables the nuclear translocation of Foxo1 in T cells (Oh et al. 2012). This shows that both physiological and pathological mediators of angiogenesis may regulate Foxo1. The increased expression of Ang-2 driven by the Ang-2/Foxo1 signalling is a key feature of vascular remodelling. Ang-2 upregulation causes pericytes to detach and migrate away from endothelial cells.

2 Retinal vasculature and neurovascular unit

The mammalian retina is delicate and requires a carefully controlled microenvironment (Klaassen et al. 2013). It has one of the highest metabolic demands in the whole body, due to the energy required by photoreceptors for visual function (Arden et al. 2005). Therefore the retinal vasculature must provide adequate perfusion to the retina to deliver sufficient metabolites and oxygen whilst not interfering with light detection.

The neurovascular unit links the retinal vasculature with retinal neurons, and consists of endothelial cells closely associated with pericytes, astrocytes and Müller cells, which regulate the growth and stability of the vascular network and maintains the inner blood-retinal barrier (BRB) (Stem & Gardner 2013; Moran et al. 2016; Park et al. 2017), the choroidal vasculature and the retinal pigment epithelium (RPE) make up what is known as the outer BRB and will be discussed later. These intimate associations allow the fine-tuning of blood flow to meet the precise metabolic demands of the neurosensory retina (Stem & Gardner 2013; Kur et al. 2012). The unique nature of the neurovascular unit contributes to the well-regulated BRB, and the role of the different cells that make up the neurovascular unit and how they maintain low rates of vascular permeability will be discussed below.

2.1 Pericytes

Extensive pericyte coverage in the retinal vasculature is believed to be associated with the tight control of vascular permeability (Armulik et al.

2011). Pericytes are intimately associated with endothelial cells with a shared basement membrane (Ribatti et al. 2011), physical attachments to the endothelium by neural cadherin (N-cadherin) junctions (Tillet et al. 2005), and gap junctions which provide an intercellular pore enabling direct cell-cell communication (Bobbie et al. 2010).

Pericytes and endothelial cells are linked by common paracrine signalling pathways which play a role in both physiological and pathological regulation of the retinal vasculature (Sweeney et al. 2016). PDGF-B mediated pericyte recruitment to the sprouting endothelium is one of the first endothelial-pericyte signalling pathways activated and is essential for development of the mature retinal vasculature (Park et al. 2017; Ogura et al. 2017; Hellström et al. 1999). Pericyte attachment to newly formed vessels requires N-cadherin, and N-cadherin knock out (KO) mice die *in utero* (Tillet et al. 2005) and antagonising N-cadherin in adult mice enhances vascular leakage (Turley et al. 2015). This indicates that N-cadherin has important roles in both development and maintaining integrity of the vasculature.

Whilst pericytes and pericyte-derived Ang-1 play an essential role in vascular development and protection from disease-associated insult (Jeansson et al. 2011; J. Zhao et al. 2015; Ogura et al. 2017), they appear to be redundant for maintaining retinal vascular quiescence (Park et al. 2017). Additionally in the retina, pericytes aren't the source of Ang-1 (Park et al. 2017). This suggests that pericytes and Ang-1 are primarily involved in the downregulation of signalling pathways that disrupt vascular

homeostasis and Ang-1 may be redundant for maintaining vascular quiescence.

2.2 Macroglia

Müller cells and astrocytes are the two types of macroglia found in the retina, where they associate with neurons to maintain normal physiological function and homeostasis (de Hoz et al. 2016). They form a direct link between the neurons and blood vessels which facilitates the transfer of nutrients and waste products and regulate blood flow in response to neuronal activity (de Hoz et al. 2016). In addition, the coupling of both Müller cells and astrocytes enhances the integrity of the BRB (Abbott et al. 2006; Tout et al. 1993) which is crucial for maintaining the carefully regulated microenvironment. Since the neurovascular unit which is only found in the brain and retina has such a carefully regulated barrier, this implies that macroglia enhance the barrier properties of the vascular endothelium beyond what can be achieved by pericytes alone.

The unique properties of the retinal vasculature carefully maintain the neural retinal microenvironment to ensure visual function. Cells of the neurovascular unit secrete paracrine mediators and directly interact with endothelial cells. Pericytes, Müller cells and astrocytes all have functional capabilities that are designed to counter vascular insults such as inflammation, oxidative stress and hypoxia. For example Müller cells have an enriched supply of anti-oxidants which have a neuroprotective effect by reducing oxidative stress, preventing damage to neurons (de Hoz et al. 2016). Pericytes also modulate the response to pro-inflammatory and permeability-inducing agents through paracrine signalling (Park et al.

2017). Whilst the cells of the neurovascular unit are able to adapt to acute stresses, chronic conditions such as diabetes override the protective activities of the neurovascular cells. This can induce an 'inflammatory switch' resulting in chronic inflammation. This manifests itself in several ways such as pericyte loss (Pfister et al. 2008; Park et al. 2014), astrocyte dysfunction (Ly et al. 2011) and secretion of pro-inflammatory and vascular permeability-inducing agents by Müller cells (Yafai et al. 2013). The pathophysiology of the retinal vasculature and the neurovascular unit and its contribution to the altered microenvironment in diabetic retinopathy will be discussed in further detail later in this chapter.

2.3 Retinal endothelial cells

2.3.1 Inter-endothelial cell junctions

The retinal vascular endothelial cell is adapted to maintain a tight barrier between the blood and the retinal tissue. It achieves this through the abscence of fenestrations, a low number of transport vesicles and the presence of tight junctions (Klaassen et al. 2013). There are three types of junctions which contribute to the blood-retinal barrier; gap junctions, adherens junctions and tight junctions (Klaassen et al. 2013) (Figure 2).

The adherens junction is one of the first intercellular junctions to form during sprouting angiogenesis (Ribatti & Crivellato 2012) and is primarily made up of VE-cadherin which joins and stabilises endothelial cells. Intercellular VE-cadherin junctions are dynamic and undergo frequent reorganisation, regulate intercellular signalling and play a key role in tight junction formation by inducing claudin-5 expression (Taddei et al. 2008).

Therefore, the formation of this initial junction between endothelial cells is crucial for producing a stable vessel wall and promoting BRB integrity.

Endothelial tight junctions are spread throughout the intercellular junction, with increased numbers of inter-endothelial tight junctions at the cell-cell interface correlating with increased control of vascular permeability (Klaassen et al. 2013). Claudins-1, 2 and 5 and occludin (Hirase et al. 1997) are the main proteins that make up the inter-endothelial tight junction. However, claudin-5 is the most highly expressed tight junction protein in endothelial cells by several orders of magnitude (Ohtsuki et al. 2007). Other endothelial tight junction proteins include junctional adhesion molecules (JAMs) and endothelial-cell specific adhesion molecule (ESAM) (Klaassen et al. 2013), however, their functions in tight junction and barrier integrity are less well understood than the claudins and occludin. The claudins, occludin, JAMs and ESAM are all linked to the cytoskeleton by the intercellular tight junction protein zonula occludens-1 (ZO-1), which regulates the tight junction protein complex and associated cytoskeleton. This in turn regulates cell morphology around intercellular junctions and maintains barrier formation (Tornavaca et al. 2015).

Endothelial tight junction proteins (occludin, claudins-1, 2 and 5) are generally not downregulated during disruption of the BRB, with claudins-2 and 5 being upregulated (Luo et al. 2011) in what may be a compensatory mechanism. However, in the mouse oxygen induced retinopathy (OIR) model, the distribution of tight junctions proteins were altered (Figure 2) and they were no longer enriched at endothelial cell junctions (Luo et al.

2011). This indicates that the localisation of tight junction proteins at the cell membrane is more important than their absolute abundance. A decrease in ZO-1, which regulates tight and adherens junction organisation is associated with severe vascular leakage (Komarova et al. 2017). This indicates that the vascular endothelium may tolerate a reduction in tight junction protein content, but the correct positioning of tight junction proteins at the endothelial cell junction and their interaction with the cytoskeleton are crucial for maintaining junctional integrity.

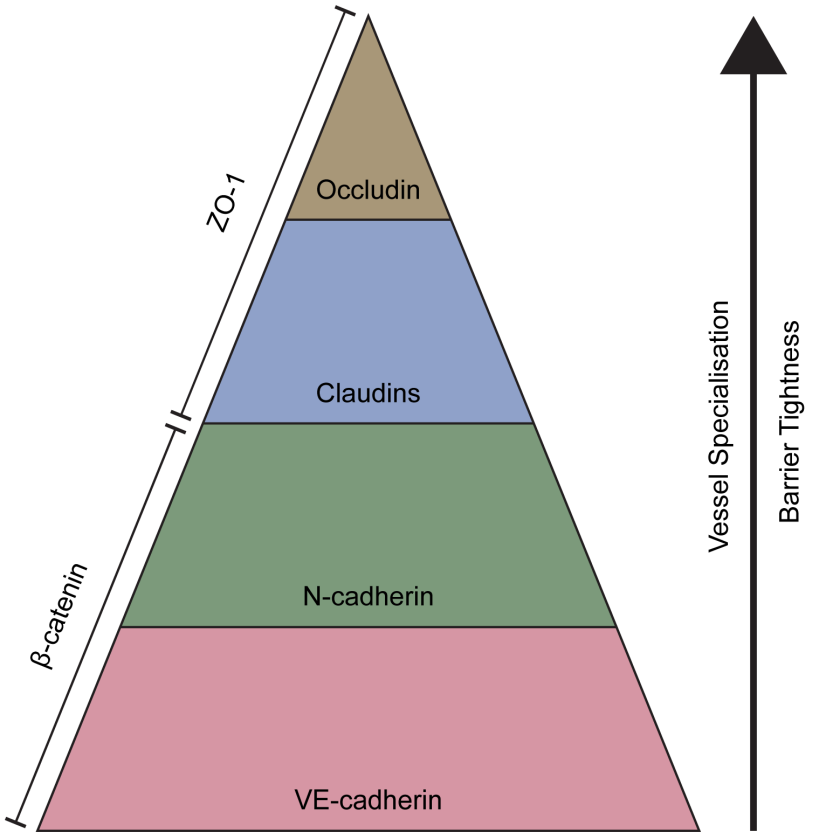

Figure 1: Hierarchy of inter-endothelial cell junctions. VE-cadherin is a ubiquitously expressed junctional protein and is essential for basal permeability and stability of the vascular endothelium. N-cadherin is expressed at endothelial-pericyte junctions, and as such is associated with tighter and more specialised vascular beds. Tight junctions are the most specialised and least widespread endothelial junction. Additionally tight junction formation is dependent on the presence of adherens junctions.

Tight junctions and adherens junctions both play important roles in maintaining the BRB, and different types of junctions exert distinct effects in the regulation of vascular permeability. Adherens junctions mediated by VE-cadherin are the first junctions to form between endothelial cells and their role in initiating the formation of other endothelial cell junctions means this can be considered a 'master regulator' of vessel stability (Figure 1) and barrier function. Downstream of VE-cadherin junction formation is N-cadherin, which mediates interactions between endothelial cells and mural cells.

The role of N-cadherin in pericyte coverage means we can consider the degree of N-cadherin mediated adherens junction formation to be linked with specialisation of the endothelium. The barrier properties of tight and adherens junctions also differ and reflect the requirements for increased control of vascular permeability depending on the vascular bed. Adherens junctions are impermeable to albumin and other large macromolecules (~70 kDa) (Villasante et al. 2007), whilst tight junctions regulate the permeability of much smaller molecules (<1 kDa) (Bazzoni 2006), and ions based on their charge (Díaz-Coránguez et al. 2017).

Whilst the cadherins are 'hierarchically' more important in regulating vascular permeability (Figure 1), tight junctions also play a crucial role in specialised vascular beds where the passage of solutes to delicate neurosensory tissue must be carefully controlled. Additionally, spatial regulation of both adherens and tight junction protein distribution is essential to their roles in maintaining endothelial barrier function, perhaps

more so than their absolute level of expression as alluded to by analysis of occludin in the mouse model of oxygen-induced retinopathy (a model of ischemia induced neovascularisation) (Barber et al. 2000).

2.3.2 Transcellular permeability

Most transport across the retinal endothelium is mediated either by membrane bound receptors or carrier proteins, and with the exception of nutrients, is energy dependent. These membrane bound transporters have distinct functions such as; the transport of nutrients, the exchange of ions and the efflux of drugs and potentially toxic molecules which diffuse across the plasma membrane (Klaassen et al. 2013). Due to the restriction of free diffusion between endothelial cells in the retinal vasculature, specific carrier proteins mediate the transport of nutrients such as hexoses, amino acids, nucleosides and other metabolic precursors across the lumen via facilitated diffusion (Zlokovic et al. 2008).

Glucose transporter-1 (GLUT-1) is probably one of the most important nutrient carriers in the retina as glucose is the most important nutrient for neuronal tissue (Chertov et al. 2011) and it is expressed on both the luminal and abluminal sides of the retinal endothelial cell (Takata et al. 1992). GLUT-1 activity is insulin-independent and is therefore dependent on blood glucose levels (Bakker et al. 2009). Asymmetrical distribution of GLUT-1 towards the abluminal side of the endothelial cell helps maintain a concentration gradient between the vasculature and retinal tissue (Zlokovic et al. 2008). When retinal endothelial cells are exposed to elevated levels of glucose, the rate of glucose transport is increased (Busik et al. 2002)

without affecting the rate of glucose consumption by endothelial cells (Busik et al. 2008). This means that in hyperglycaemic conditions such as diabetes, increased glucose transport across the endothelium exposes retinal cells such as photoreceptors to high glucose levels, which contributes to the pathology of diabetes, and that hyperglycaemia has effects beyond the vascular endothelium.

Larger macromolecules cross the retinal endothelium through specialised vesicles, known as caveolae, in a receptor-dependent or -independent fashion (Klaassen et al. 2013). Unlike other vascular beds, caveolae are the only organelle which facilitate transcellular transport in the retinal endothelium (Hofman et al. 2000). Whilst the paper by (Hofman et al. 2000) showed by electron microscopy that VEGF induced formation of caveolae but not fenestrae in the retinal endothelium, one cannot rule out the possibility that other growth factors may induce the formation of fenestrae, however, this is unlikely to occur. Plasmalemma vesicle-associated protein (PLVAP) is essential for the formation of caveolae (Figure 2), where it plays an important role in forming the structure of the caveolae (Guo et al. 2016). It is upregulated in diseases associated with angiogenesis and vascular leakage (Guo et al. 2016). Since VEGF plays a key role in mediating vascular permeability, intravitreal administration of VEGF to the mouse retina elevated transcellular vascular permeability through increased PLVAP dependent caveolae transport rather than fenestration or vesiculo VVO formation (Hofman et al. 2000). This indicates that caveolae mediate transcellular permeability in the retinal vasculature in both physiological and pathological conditions.

Increased permeability of the retinal vasculature can be increased through paracellular and transcellular routes. In diabetic retinopathy perturbations occur to regulators of both routes of vascular permeability (Navaratna et al. 2007), with VEGF promoting both transcellular permeability through PLVAP upregulation (Wisniewska-Kruk et al. 2016) and the phosphorylation of tight junction proteins (Antonetti et al. 2012) (Figure 3). Whilst the contribution of adherens and tight junction loss to increased vascular permeability has been more widely investigated, the role of transcellular permeability should not be discounted and is likely to play an equally important role.

2.4 The choroidal vasculature and the outer-BRB

In contrast to the retinal vasculature, the choroid is much more extensively vascularised and the choroidal vasculature is leaky and fenestrated (Aird 2007). Whilst inter-endothelial junctions regulate vascular permeability in the retinal vasculature and makes up the inner-BRB, the outer-BRB is regulated by the RPE (Fig 2). Tight junctions between RPE cells and the presence of specific transporters control the passage of water, solutes and other molecules into the retina, which is vital for the maintenance of retinal homeostasis (Benedicto et al. 2017).

Whilst there is some evidence of outer-BRB disruption in diabetes, such as dysfunction of the outer retina (Samuels et al. 2014), there is limited evidence of outer-BRB derived leakage only occurs very late in the streptozotocin mouse, with evidence of leakage occurring at earlier time-points in the diabetic rat (Simó et al. 2010). Examination of the RPE in streptozotocin-induced diabetic mice 2-months after diabetes induction and

mice fed a high fat diet for 5 weeks revealed no changes to the integrity of the RPE (data not shown). Therefore this avenue of inquiry was not further pursued. Examination of the RPE in other models of diabetes, which present a more severe phenotype may have identified a disruption to the RPE which may have identified scope for further research. Evidence from diabetic patients shows that vascularisation of the choroid is decreased in diabetic retinopathy (Gupta et al. 2017). This could contribute to oxidative stress in the retina as the capacity of the choroidal vasculature to provide nutrition to the retina is likely to be diminished.

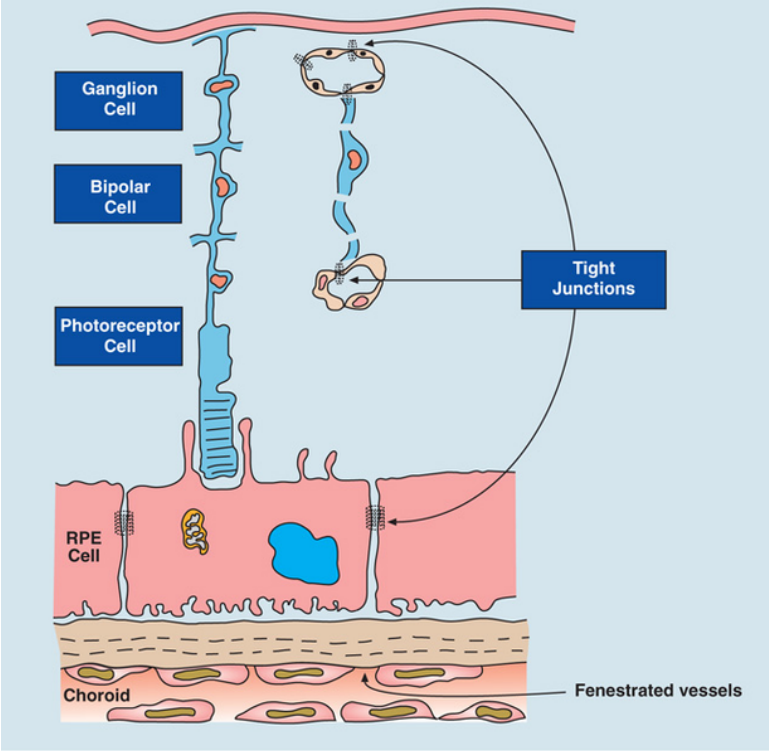

Figure 2: a schematic representing the blood-retinal barriers (Forrester & Xu 2012). Tight junctions regulate the passage of various molecules into the retina, the intra-retinal vasculature contains tight junctions between endothelial cells, whilst the RPE has tight junctions between cells which regulates the flow of molecules derived from choroidal vasculature.

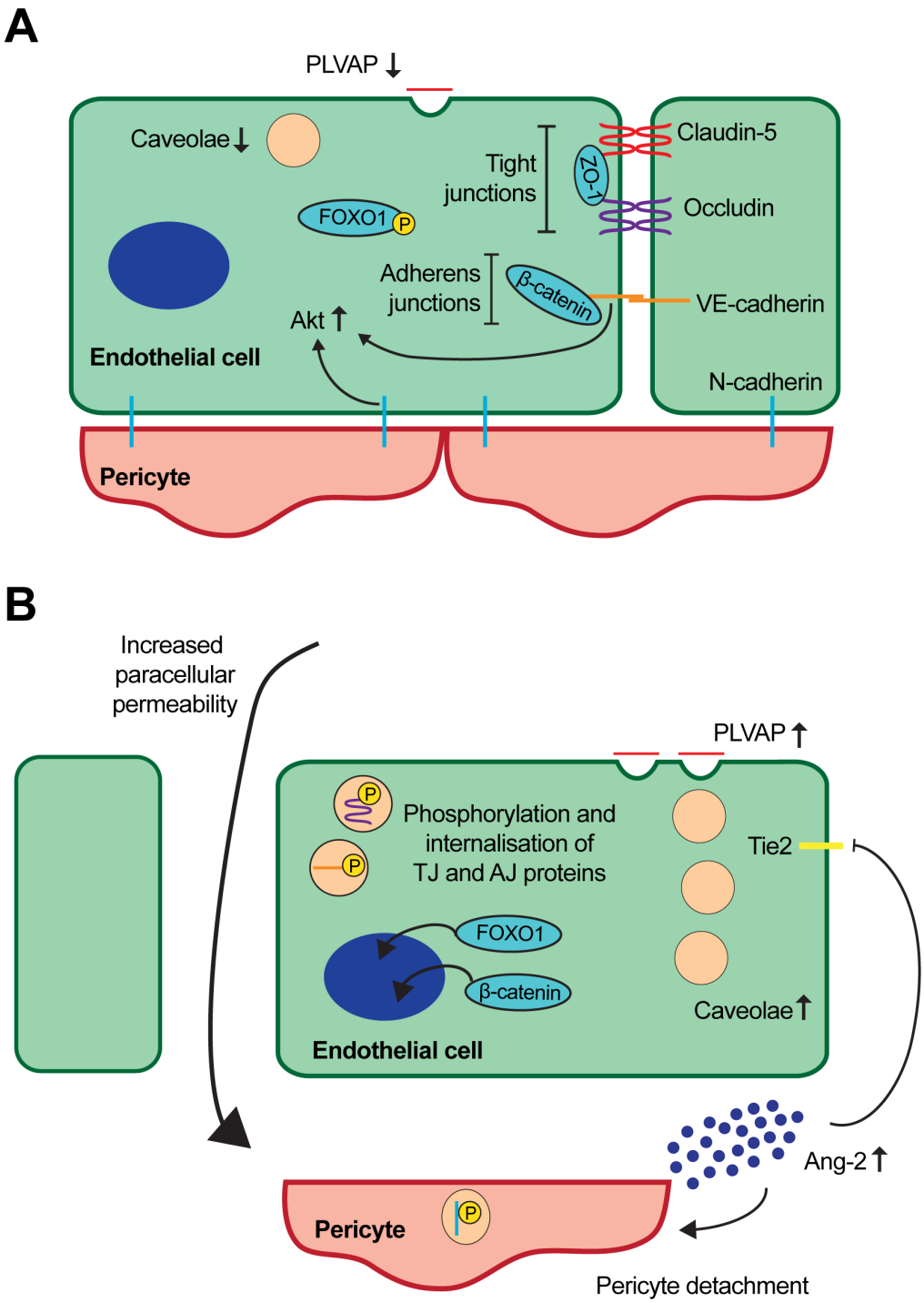

Figure 3: The quiescent and active vasculature. A: the quiescent vasculature has intact endothelial junctions and low levels of caveolae and associated low levels of PLVAP expression, a key structural protein required for cavaeolae function leading to reduced paracellular and transcellular permeability. B: the activated endothelium has weakened endothelial junctions and increased numbers of caveolae, which increases permeability. This is due to pericyte detachment and altered gene expression.

3 Diabetes and diabetic retinopathy

Diabetes manifests itself either through the loss of insulin production following destruction of pancreatic β-cells (Type 1 Diabetes) or development of insulin resistance (Type 2 Diabetes). Both types of diabetes lead to elevated blood glucose, which has a range of impacts on different organs of the body leading to increased morbidity and mortality. Diabetes is associated with multiple microvascular and macrovascular complications, but diabetic retinopathy is the most common complication associated with the microvasculature and the retina.

One third of the 246 million diabetic patients have some signs of diabetic retinopathy with a third of these having signs of severe retinopathy (Cheung et al. 2010). Diabetic retinopathy manifests itself in two stages, the proliferative and non-proliferative stage. The first is the early non-proliferative diabetic retinopathy (NPDR), which is characterized by microaneurysms, lipid exudates, haemorrhage, nerve fibre damage, basement membrane thickening, pericyte dropout, vascular regression and formation of tortuous vessels. Proliferative diabetic retinopathy (PDR) typically follows NPDR and is characterized by neovascularization, retinal detachment and decreased visual acuity. Diabetic macular oedema (DMO) may occur at either stage and is associated with vascular leakage and retinal thickening (Antonetti et al. 2012)

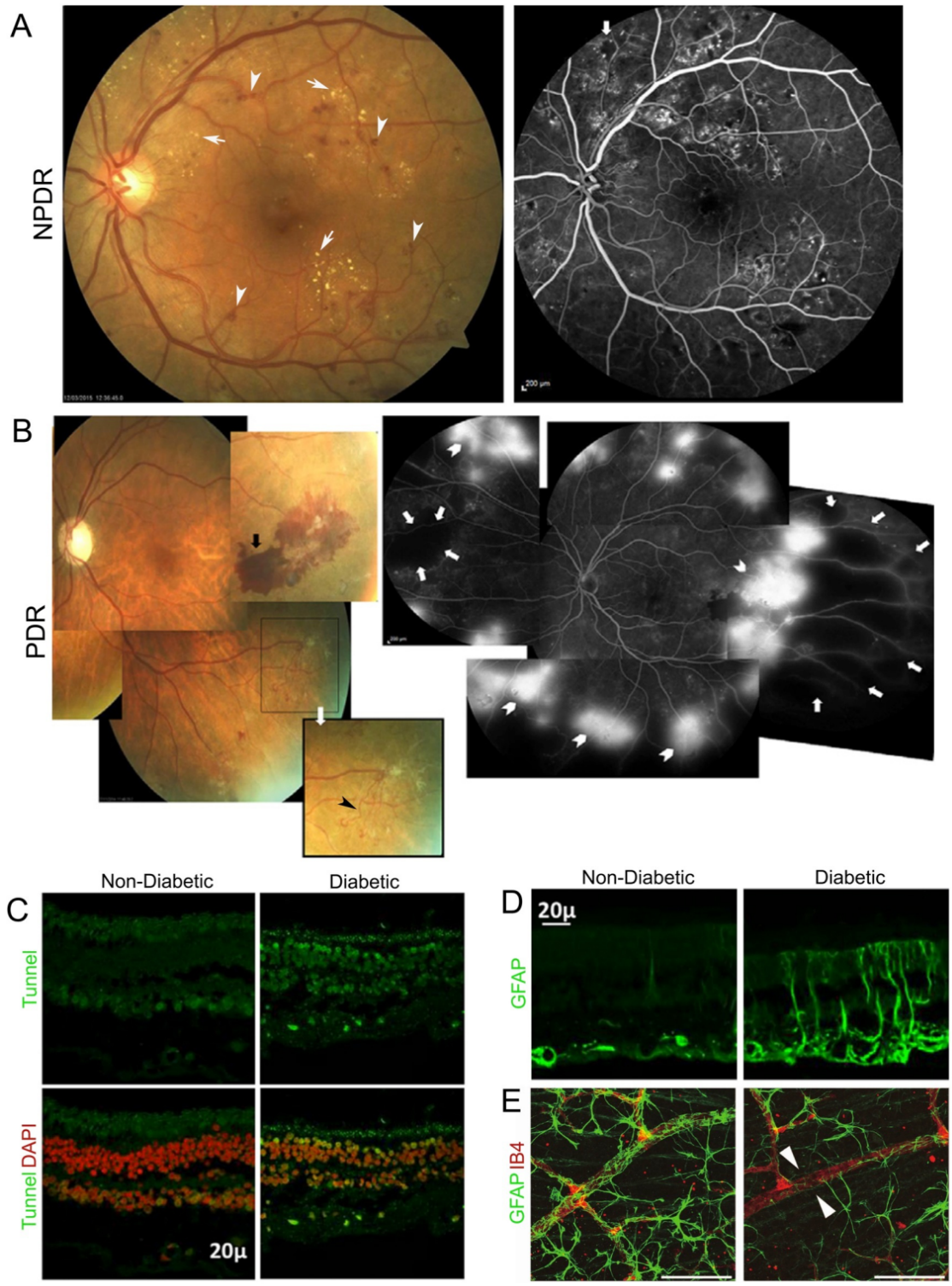

Figure 4: The multiple stages and mechanisms of pathophysiology of diabetic retinopathy. Adapted from (Stitt et al. 2016) & (Ly et al. 2011). A: Fundus image (left) and fluorescein angiogram (right) from a patient with NPDR. The fundus image reveals the presence of microaneurysms (arrowheads) and areas with inflammatory infiltrates (arrows). Fluorescein angiogram shows the microaneurysms more clearly

due to the extravasated fluorescein and unperfused ischemic areas (white arrow). B: Fundus image (left) and fluorescein angiogram from a patient with PDR. During PDR, the severity of the vascular complications have increased with increase haemorrhage (black arrow) and the formation of tortuous neovessels (black arrowhead). The fluorescein angiogram highlights increased areas of hypoxia (white arrow) with increased areas of vascular leakage (white arrowhead) compared to the NPDR patient in A. C: Sections from non-diabetic and diabetic donor eyes analysed for apoptosis reveals an increased number of apoptotic cells in the diabetic retina. D: Sections from non-diabetic and diabetic donor eyes stained for GFAP reveal increased GFAP staining in the Müller cells, which is indicative of reactive gliosis and retinal stress. E: GFAP staining in a flatmounted control and diabetic rat retina (Ly et al. 2011). Diabetes reduces the total number of astrocytes and the number of interactions with the retinal vasculature.

Progression in the severity of diabetic retinopathy is common in the long term. In a 14 year population based study to measure the progression and incidence of diabetic retinopathy, 86% of patients had an increased severity grading in retinopathy, from which 26% progressed to DMO and 37% progressed to PDR (Klein et al. 1998). It is possible that some patients did not progress to PDR due to a satisfactory management of diabetic retinopathy. The high incidence of diabetic retinopathy in diabetic patients and the high rate of progression in the severity of diabetic retinopathy show that the clinical relevance of diabetic retinopathy is becoming increasingly important since the number of diabetic patients is increasing, which is likely to lead to a greater number of patients at risk of vision loss. This highlights a need for improved understanding of pathophysiology, identification of new therapeutic targets and improved clinical management of patients.

3.1 Current therapeutic strategies

A therapeutic target is anything within a living organism with which can be modulated resulting in a functional change in the context of a given disease. For example, VEGF is a therapeutic target for diabetic retinopathy and many other diseases (Zaghloul et al. 2009), and its activity can be modulated, for example by blocking antibodies which prevent it functioning as a signalling molecule. Since VEGF blockade has several side effects and limitations which will be discussed below, it can be considered a therapeutic target with limited (although still potent) efficacy.

VEGF blockade has been the best therapeutic strategy to offer improvements to DR patients vision, compared to laser therapy and corticosteroids (Brown et al. 2013; Elman et al. 2012; The Diabetic Retinopathy Clinical Research Network 2015). Aflibercept is the latest anti-VEGF agent, which is the current first line therapeutic for the management of diabetic retinopathy (Heier et al. 2016). Despite the benefits of anti-VEGF therapies there are several side effects which result from this therapy, including intraocular inflammation, retinal detachment, intraocular pressure elevation, haemorrhage and systemic effects (Falavarjani & Nguyen 2013). Additionally VEGF blockade is not always effective in the long term, with 46% of patients requiring laser photocoagulation after 2 years and 40% seeing no improvement in visual acuity (Elman et al. 2012). Another limitation of VEGF blockade for the treatment of DR is that it can only be used when the vascular symptoms of DR appear and not before. Therefore a lack of treatments for the non-proliferative stage of DR present a major unmet clinical need.

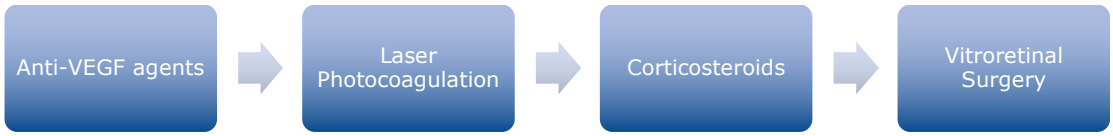

Figure 5: Current therapeutic strategies for the management of diabetic retinopathy. VEGF blockade is the current first line therapeutic used in the clinic after evidence of macular oedema is observed. Currently there is no therapeutic which can be administered prior to the development of oedema or neovascularisation. Laser photocoagulation is a second line therapeutic used in patients who don't respond, develop resistance or have other contraindications which prevent the use of VEGF blockade. Vitroretinal surgery is only used in advanced cases.

Laser photocoagulation, is the current second line therapeutic used in the clinic. The aim of laser photocoagulation is to stabilise vision rather than offer improvements. This can only be utilised after the disease progresses into the macula. Vitreoretinal surgery is used to manage the late stage of the disease where blood leakage occludes vision and tissue scarring is present.

Corticosteroids have been used to target increased inflammation typically associated with diabetic retinopathy. Despite the important role of inflammation in the pathology of diabetic retinopathy, treatment with steroids has shown limited success with only temporary benefits and various side effects including cataracts and glaucoma (Grosso & Panico 2009).

3.2 Metabolic Changes

Glucose uptake by endothelial cells from the blood is mediated by GLUT-1, an insulin independent glucose transporter (Bakker et al. 2009). When diabetes is poorly controlled, intracellular glucose levels increase in line with blood glucose levels above normal physiological levels. This leads to increased glycolysis and accumulation of intermediate glucose metabolites and altered glucose metabolism (Giacco & Brownlee 2010).

3.2.1 Altered glucose metabolism

During diabetes there is increased activity of the polyol pathway, where glucose and its accumulated metabolites are converted to sorbitol by aldose reductase which utilises nicotinamide adenine dinucleotide phosphate (NADPH) (Mathebula 2015). NADPH acts as a reducing agent and is required for the regeneration of cellular antioxidants.

During diabetes, the hexosamine biosynthesis pathway is increased due to fructose 6-phosphate accumulation (Buse 2006). This leads to O-Linked β-N-Acetylglucosamine (O-GlcNAc) post-translational modifications to proteins (Semba, R., et al. 2014). This can modulate gene expression and signalling, by altering transcription factors such as specificity protein 1 (Sp-1) and inhibiting eNOS activity (Giacco & Brownlee 2010).

3.2.2 AGE accumulation

Accumulation of intermediate glucose metabolites and altered glucose metabolism, promotes the formation of advanced glycation end products (AGEs) resulting in AGE deposition on proteins (Yamamoto, Y. & Yamamoto,

H., 2012). AGEs can alter the vasculature by several different mechanisms such as modifying proteins involved in gene transcription, modifying ECM proteins and integrin signalling, modification of circulating proteins and activation of various AGE receptors, with the most important being the receptor for advanced glycation end products (RAGE) (Stirban et al. 2014). STAT3, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1) and Forkhead box (Foxo) transcription factors are all activated downstream of RAGE activation (Stirban et al. 2014), which shows this is to be an important mediator of pro-inflammatory signalling. RAGE is widely expressed on many cell types and mediates the early stages of pathology in diabetic retinopathy (Barile et al. 2005), by promoting oxidative stress, inflammation and apoptosis (Stitt 2010). *In-vitro* studies have demonstrated that advanced glycation end product – bovine serum albumin (AGE-BSA) induces endothelial cell proliferation and VEGF production (Mamputu & Renier 2004). However, long term exposure to AGE induced signalling in the diabetic retina may eventually lead to endothelial cell death induced by inflammation and oxidative stress.

Altered glucose metabolism has a wide range of effects on the retina and the retinal vasculature, including altered gene expression, inflammation and oxidative stress. Reciprocal interactions between inflammation and oxidative stress exacerbate aberrant signalling pathways, which contribute to hypoxia and vascular dysfunction in diabetic retinopathy.

3.3 Pathophysiology

Since the primary symptom of diabetes is elevated blood glucose it is easy to assume that endothelial cell dysfunction is the first event in the pathogenesis of diabetic retinopathy and other microvascular complications of diabetes (Bakker et al. 2009). Whilst diabetic retinopathy has a clear vascular phenotype, neuroretinal symptoms appear before vascular complications arise (Antonetti et al. 2006). The pathophysiological features of diabetic retinopathy are summarised in (Fig. 5), which highlights how inflammation, neurovascular changes and vascular dysfunction are related and contribute to diabetic retinopathy. All of these features of diabetic retinopathy will be discussed in further detail later in this thesis. Considering how pericytes, glial and endothelial cells are intimately associated within the neurovascular unit, it's unsurprising that endothelial cell dysfunction may not be the first event in the pathophysiology of diabetic retinopathy.

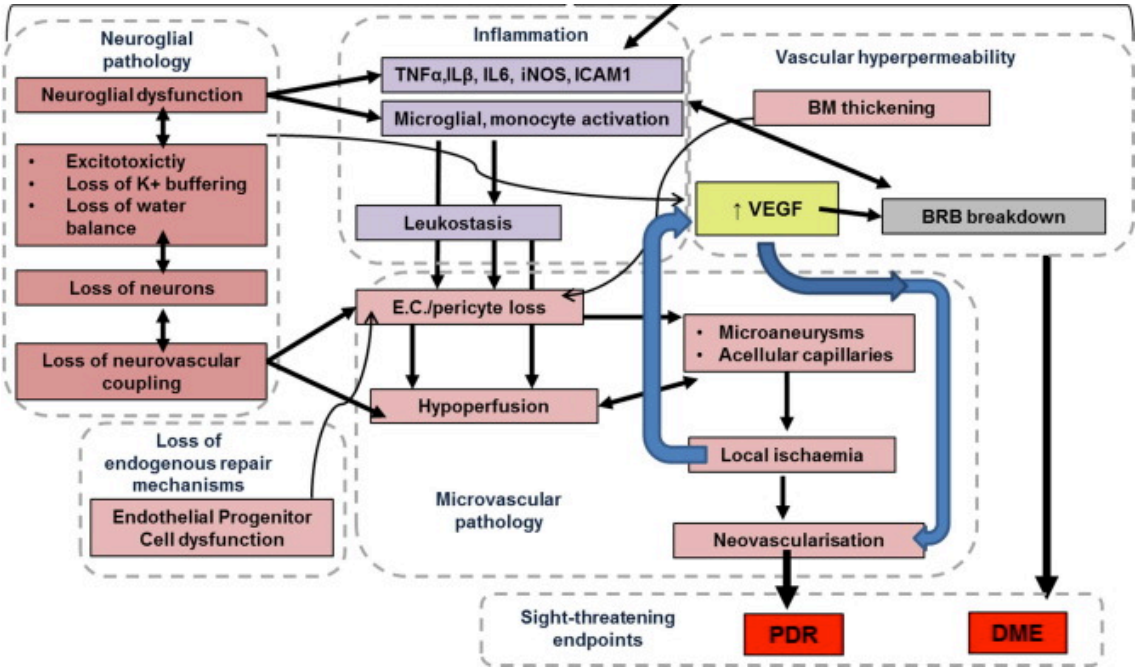

Figure 6: A summary of the mechanisms of pathophysiology in diabetic retinopathy (Lechner et al. 2017). This highlights how multiple aspects of the pathophysiology of diabetic retinopathy are interlinked and act together to increase the severity of this disease leading to the risk of vision loss.

3.3.1 Astrogliosis

Astrocyte dysfunction occurs prior to the development of Müller cell gliosis in the streptozotocin induced diabetic rat (Ly et al. 2011). Additionally retinal ganglion cell death (Kern & Barber 2008), hypoxia and vascular leakage in the superficial plexus (Ly et al. 2011) occur early in diabetic retinopathy. In diabetic rats, reduced astrocyte number, processes and connexin-43 expression occurred early in diabetes (Ly et al. 2011). This implies that astrocyte dysfunction may be responsible for disruption of the neurovascular unit in this plexus.

High glucose induces astrocyte proliferation *in vitro* (Shin et al. 2014), which may drive a transient increase in astrocyte number early in diabetic

retinopathy (Ly et al. 2011). Similar to Müller glia, this could be a protective response to increased retinal stress to protect retinal neurons. High glucose alters astrocyte migration and morphology (Shin et al. 2014) which may explain the reduction in astrocytic processes which are involved in the interaction with endothelial cells (Figure 3 E). During astrogliosis, there is also an increase in secreted inflammatory cytokines such as IL-6 and monocyte chemoattractant protein 1 (MCP-1) (Sorrentino et al. 2016), which is mediated by oxidative stress-induced p38 mitogen activated protein kinase (MAPK) signalling (Nahirnyj et al. 2013). Altered astrocyte proliferation and morphology was shown to be attenuated by anti-oxidant treatment *in vitro* (Shin et al. 2014). This shows that oxidative stress appears to be a key driver of astrocyte dysfunction in diabetes. Astrocyte biology is also influenced by Ang-2 in diabetes. Upregulation of Ang-2 expression in diabetic retinopathy promotes astrocyte apoptosis via integrin signalling (Yun et al. 2016). Therefore in addition to its role in pericyte loss, Ang-2 also contributes to a reduction in astrocyte number. This shows that multiple aspects of the diabetic microenvironment alter astrocyte behavior and their interactions with the vasculature.

3.3.2 Müller cell gliosis

In diabetic retinopathy Müller cells undergo various changes such as; increased glial fibrillary acidic protein (GFAP) expression, reduced glutamine synthetase expression, disrupted neurotransmitter uptake and cytokine secretion (Kur et al. 2012). GFAP expression is associated with reactive gliosis, which occurs in response to retinal stress (Bringmann & Wiedemann 2012). Reactive gliosis in Müller cells occurs later in diabetic retinopathy

than astrocyte dysfunction, and is associated with reduced photoreceptor activity (Ly et al. 2011). In diabetes, AGE/RAGE signalling in Müller cells (Stitt et al. 2016) and hypoxia (Xin et al. 2013) promotes Müller cell gliosis. Whether hypoxia occurs prior to gliosis or secondary to inadequate blood flow due to reduced interaction between neurons and the vasculature (neurovascular coupling) is is unknown. Regardless of the mechanism, hypoxia induced signalling is a key mechanism for Müller cell induced vascular permeability (Xin et al. 2013; Wang et al. 2010), either through the release of growth factors or signaling through cell-cell junctions, possibly connexin-43 (Bobbie et al. 2010).

During acute periods of retinal stress, the secretion of pro-inflammatory mediators such as MCP-1 and tumour necrosis factor-α (TNF-α) during reactive gliosis helps recruit monocytes and microglia to areas of retinal stress (Bringmann & Wiedemann 2012). Sustained secretion of these pro-inflammatory cytokines during extended periods of gliosis leads to chronic inflammation (Yu et al. 2015). Müller cells also secrete pro-angiogenic and vascular permeability inducing agents VEGF (Bai et al. 2009), fibroblast growth factor 2 (FGF-2) (Yafai et al. 2013) and angoipoietin like 4 (Angptl-4) (Xin et al. 2013). VEGF and FGF-2 are neuroprotective and promote photoreceptor survival, by activating pro-survival signaling pathways (Yafai et al. 2013). However, sustained secretion of these growth factors promotes chronic inflammation and vascular leakage. Additionally disruptions to nutrient and waste exchange may contribute to the induction of oxidative stress in photoreceptors (Du et al. 2013), exacerbating the chronic inflammatory microenvironment.

3.3.3 Pericyte loss

Pericyte loss in diabetic retinopathy is one of the first vascular defects to occur (Stitt et al. 2016). Whilst the ratio of pericytes to endothelial cells in the retina is one of the highest throughout the vasculature (Cuthbertson & Mandel 1986), even small reductions in pericyte coverage may lead to vascular dysfunction (Pfister et al. 2008; Hammes et al. 2004). Therefore even minor mechanisms of pericyte loss may be clinically relevant to diabetic retinopathy, as the high pericyte-endothelial cell ratio may be required for the maintenance of the retinal vasculature.

Several publications have reported that pericyte loss in DR is mediated by Ang-2 (Hammes et al. 2004), possibly acting through multiple mechanisms. For example, Ang-2 may induce pericyte loss by inducing pericyte migration (Pfister et al. 2008) or apoptosis (Park et al. 2014).

Pericyte loss leads to impaired BRB integrity, (Park et al. 2017) increased risk of developing a pro-inflammatory state in the endothelial cells such as upregulation of *IL6* and *TNFa* expression, leukocyte adhesion and infiltration (Ogura et al. 2017). As pericytes play a key role in the neurovascular unit by linking endothelial cells with astrocytes and glial cells, a loss of pericyte coverage may be expected to play a key role in disruption of the neurovascular unit (Klaassen et al. 2013).

3.3.4 Endothelial cell dysfunction

In diabetic retinopathy, disruption of the quiescent and selectively permeable endothelium (Fig. 2 B) occurs primarily due to paracrine

mediators from two sources, the cells of the neurovascular unit (Figure 6) and circulating leukocytes that adhere to the vessel wall. Disruption of the neurovascular unit leads to insufficient blood flow (de Hoz et al. 2016), reduced barrier integrity (Gardner et al. 1997), endothelial cell inflammation and monocyte recruitment (Park et al. 2017; Ogura et al. 2017). Leukocyte adhesion molecules are upregulated following the release of inflammatory mediators from cells of the neurovascular unit (Yu et al. 2015). Impaired neurovascular coupling and the secretion of pro-inflammatory factors promote pro-inflammatory and permeable conditions in the retinal vasculature.

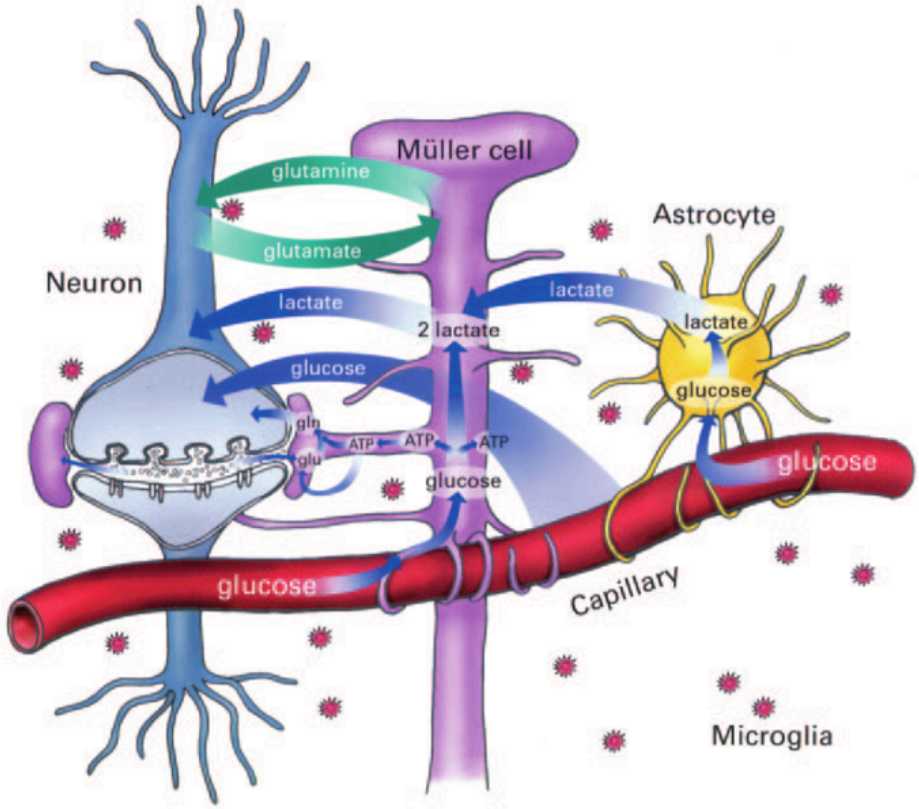

Figure 7: The neurovascular unit of the retina (Antonetti et al. 2006). Light sensing retinal neurons are physically linked to the retinal vascular endothelium by Muller cells and Astrocytes, which dynamically regulate blood flow according to the metabolic demands of the retina.

3.3.4.1 Disruption to endothelial cell junctions

Endothelial adherens junctions are disrupted by VEGF dependent VE-cadherin phosphorylation and internalization (Gavard & Gutkind 2006) and MMP mediated proteolysis of the extracellular domain of VE-cadherin (Navaratna et al. 2007). These two mechanisms indicate that multiple mechanisms are involved in the breakdown of the BRB in diabetic retinopathy. Aside from its role in maintaining inter-endothelial cell junctions, VE-cadherin associates with VEGFR2 (Giannotta et al. 2013) and the TGF-β receptor complex (Rudini et al. 2008) which suppresses both receptors signalling pathways by tethering these receptors in an inactive conformation. Additionally VE-cadherin prevents β-catenin entering the nucleus and inactivates Foxo1 by phosphorylation (Taddei et al. 2008). Therefore disruption of VE-cadherin junctions sensitises endothelial cells to various vascular permeability inducing agents and the formation of intercellular VE-cadherin interactions maintain vascular quiescence and act as a negative regulator of BRB breakdown (Klaassen et al. 2013).

Adherens junction stability is important for formation and maintenance of tight junctions (Tietz & Engelhardt 2015). Disruption of the VE-cadherin and N-cadherin junctions leads to the release of β-catenin, activation of the Foxo1 transcription factor (Giampietro et al. 2012; Taddei et al. 2008) and inhibition of claudin-5 transcription (Taddei et al. 2008). N- and VE-cadherin have an additive effect on β-catenin and Foxo1 transcriptional inhibition (Giampietro et al. 2012) which means disruption of VE-cadherin junctions will exacerbate vessel destabilization due to reduced N-cadherin signalling induced by pericyte loss. Inhibition of Foxo1 reduced pericyte loss and

endothelial cell apoptosis in diabetic rats (Behl et al. 2009) which indicates that this is an important mechanism for diabetes induced vascular dysfunction. Since Ang-2 is a transcriptional target of Foxo1 (Kim et al. 2016), this suggests that vessel destabilization progresses through a positive feedback mechanism, in which Ang-2 induced Foxo1 activation is a key component.

MMP release and the resulting proteolytic degradation of the ECM is a key step in vascular remodeling. Whilst MMP mediated degradation of VE-cadherin has been shown to contribute to diabetes-induced vascular permeability (Navaratna et al. 2007), the role of MMP mediated tight junction proteolysis has not been conclusively proven in diabetes. However, MMPs have been shown to degrade tight junctions *in vitro* (Giebel et al. 2005) and at the blood-brain barrier *in vivo* (Yang et al. 2007). This may mediate the cleavage of the extracellular domains of the tight junction complex at the cell membrane and result in tight junction breakdown.

3.3.4.2 increased transcellular permeability

During diabetes, permeability is increased through the weakening of endothelial cell junctions and increased rates of transcellular permeability. PLVAP mediates VEGF-induced increases to vascular permeability in the retinal (Wisniewska-Kruk et al. 2016) and tumour vasculature (Strickland et al. 2005). In addition to its role in transcellular transport, PLVAP is also involved in leukocyte transmigration (Keuschnigg et al. 2009), where it has been shown to undergo dynamic redistribution following the pattern of transmigrating leukocytes within caveolae. Whilst the function of PLVAP in

leukocyte transmigration is unknown, it could be involved in the entry of leukocytes into caveolae following attachment to the endothelium. Both mechanisms of increased permeability may be linked, as leaky endothelial cells appear to be both claudin-5 deficient and positive for PLVAP (Zhou et al. 2014).

In the normal retina, the level of caveolae is low (Figure 2), which suppresses transcellular permeability. The vitamin D transporter, major facilitator superfamily domain containing 2A (MFSD2A) is associated with the suppression of transcytosis by altering the lipid composition of the plasma membrane, leading to a reduction in the level of caveolae (Chow & Gu 2017). Although no mechanistic link has been identified, vitamin D deficiency is associated with an increased risk of diabetic retinopathy (Luo et al. 2017), the absence of MFSD2A or its impaired activity may contribute to increased trans-endothelial permeability in diabetic retinopathy.

3.3.4.3 leukocyte recruitment

Following the activation of pro-inflammatory signalling, initial leukocyte recruitment is mediated by expression of P-selectin which allows leukocytes to adhere to the vessel wall before binding to intercellular adhesion molecule 1 (ICAM-1) (Joussen et al. 2001) and vascular cell adhesion molecule 1 (VCAM-1) (Iliaki et al. 2009). Diabetes-induced accumulation of AGEs and reactive oxygen species (ROS) have been shown to mediate leukocyte adhesion (van der Wijk et al. 2017), due to ICAM-1 and VCAM-1 upregulation through pro-inflammatory and oxidative stress induced signalling pathways (Tang & Kern 2011). Blockade of ICAM-1 (Joussen et al. 2001) or VCAM-1 (Iliaki et al. 2009) or their respective ligands cluster of

differentiation (CD)18 and CD49 prevents leukocyte adhesion and reduces inflammation and breakdown of the vascular endothelium. Since infiltrating monocytes, unlike microglia, remain in close proximity to the retinal vasculature (Stitt et al. 2016), it implies that recruited monocytes mediate inflammation around the retinal vasculature rather than at other sites in the diabetic retina.

3.3.4.4 endothelial cell apoptosis

Endothelial cell apoptosis is a key event in the development of diabetic retinopathy and has been observed in experimental models of diabetes (Joussen et al. 2009). Overexpression of the anti-apoptotic gene B-cell lymphoma 2 (Bcl-2) in endothelial cells inhibited microvascular lesions in diabetic mice (Kern et al. 2010). This indicates that endothelial cell apoptosis is a key event in the development of microvascular dysfunction, at least in rodent models of diabetic retinopathy. In post-mortem tissue taken from diabetic patients who had no symptoms of diabetic retinopathy (Stitt et al. 2016), there was an increase in the number of apoptotic cells (Figure 3 C) in patients with diabetes compared to non-diabetic donors. Whilst this did not detect whether apoptosis was occurring in endothelial cells specifically, it does indicate that apoptosis in the retina occurs prior to the later stages of diabetic retinopathy in humans.

Several mediators of endothelial apoptosis have been identified, including ROS (Castilho et al. 2012), inflammatory cytokines (Busik et al. 2008) and AGEs (McDonald et al. 2009). VE and N-cadherin activate Akt signalling (Giampietro et al. 2012) which promotes endothelial cell survival. Therefore

the disruption of VE and N-cadherin junctions attenuates pro-survival signalling in endothelial cells and increases their susceptibility to apoptotic signals.

Endothelial cell apoptosis leads to the formation of unperfused acellular capillaries, which in combination with reduced flow due to impaired glial-vascular coupling leads to the development of retinal ischemia. This is an essential requirement for the generation of pathological neovascularisation in PDR.

4 Aims of the Thesis

In humans, progression to PDR is associated with severe vision loss resulting from severe vascular changes, but this feature of diabetic retinopathy is not observed in animal models of diabetes. Whilst this limits the usefulness of animal models for studying this late stage of diabetic retinopathy, the lack of therapeutics that prevent the progression of pre-proliferative diabetic retinopathy presents a major unmet clinical requirement. As discussed earlier in this thesis, targeting VEGF has potential side effects, which limits the effectiveness of VEGF as a therapeutic target, especially as intervention can only take place after some disease progression has occurred. Therefore, the primary aim of this thesis is to identify potential new ways of treating the early stages of diabetic retinopathy by identifying new therapeutic targets, which are more effective and with fewer side effects than VEGF. In order to do this, one must investigate factors which are altered in diabetes, the effect they have at the cell and molecular level, the effect of inhibiting and otherwise altering their activity and how these alterations alter the course of the disease.

To study diabetic retinopathy, we have used the streptozotocin induced diabetic mouse, a widely used model of type 1 diabetes (Robinson et al. 2012). Whilst a common criticism of rodent models of diabetes is that they fail to model the sight threatening late stages of diabetic retinopathy, this feature fits well with the aim of this research. The use of this animal model of diabetes will be useful for investigating factors that are altered during the early stages of diabetic retinopathy. Linking changes to the transcriptome and proteome to specific pathophysiological changes in diabetic retinopathy

will be important to understanding how different factors contribute to the severity of diabetic retinopathy and whether they have the potential to act as therapeutic targets.

In addition to the *in vivo* work we also use the mouse metatarsal assay (Deckers et al. 2001; Song et al. 2015), an *ex vivo* model of angiogenesis to study angiogenesis, and the vasculature in response to diabetic insults such as hyperglycaemia and AGE-BSA. Whilst this model is limited in comparison to various *in vivo* systems to investigate the vasculature and diabetic retinopathy, it enables a robust and relatively quick method to investigate the response of various insults on a model of the vasculature.

Throughout the course of this research, several factors were identified; ANGPTL2, ANGPTL6 and LRG1, which were increased in the retinas of diabetic mice compared to controls. Therefore, I hypothesised that these factors would contribute to the severity of diabetic retinopathy, whilst the absence or inhibition of these factors would ameliorate the severity of diabetic retinopathy in the experimental models previously described.

Chapter 2 Materials and Methods

1 Molecular and Cell Biology Techniques

1.1 Metatarsal assay

The metatarsal assay is an *ex-vivo* model of angiogenesis in a complex multicellular environment which, unlike similar assays such as the aortic ring, does not require the addition of exogenous growth factors (Deckers et al. 2001; Song et al. 2015). Metatarsal bones were isolated from C57Bl/6 mice (Harlan) at E16-18. Following decapitation, hindlimbs were removed, tissue and skin was stripped from the hindlimb and metatarsal bones were dissected and separated. Excess tissue was carefully removed and isolated metatarsals were kept in ice cold PBS with 10% foetal bovine serum (FBS) until plating. Isolated metatarsals were individually cultured in a 24 well tissue culture plate (Thermo Scientific), coated with 0.2% gelatin (Sigma Aldrich). Metatarsals were initially grown in 150 µL of α-mem with 10% FBS and 100 U/mL of penicillin-streptomycin (Invitrogen). After 3 days the medium was replaced with 250 µL of fresh medium with or without treatments and refreshed every 48 hours. Metatarsals were cultured for 12 days, with fresh media being added every 2 days of culture. Metatarsals were then either fixed in 4% paraformaldehyde (PFA) for 30 minutes for immunofluorescence studies, or RNA was extracted using the RNeasy Micro RNA kit (Qiagen), according to the manufacturers instructions.

Angiogenesis was quantified by immunofluorescent labelling of vessel sprouts. Metatarsals were blocked with 10% bovine serum albumin (BSA) (Signma Aldrich) and 0.1% Triton in phosphate buffered saline (PBS) for 1 hour at room temperature, followed by incubation with appropriate primary

antibodies ((Rat anti-CD31: 1:100 (BD Biosciences), Rabbit anti-neural/glial antigen 2 (NG-2): 1:200 (Millipore), Mouse anti-α-smooth muscle actin (SMA)-cyanine 3 (Cy3): 1:400 (Sigma Aldrich), Rat anti-PLVAP: 1:100 (BD Biosciences)) in 5% BSA with 0.1% Triton in PBS, overnight at 4 ^{o}C. This was followed by three washes with 0.1% Tween-20 in PBS for 15 minutes. All secondary antibodies were purchased from Invitrogen, raised in donkey and conjugated to alexa-fluor-488, -555 or -647. Appropriate secondary antibodies were incubated at 1:1000 in 5% BSA with 0.1% Triton in PBS for 2 hours at room temperature. This was followed by three washes with 0.1% Tween-20 in PBS for 15 minutes.

To quantify vascular outgrowth in the metatarsal assay, images of CD31 staining were captured using an Olympus Stereo microscope at 2x magnification. Images were analysed using AngioSys 1.0 (Cellworks) to quantify the total amount of vessel sprouting and branching. Vessel sprouting was defined as the total length of all the vessel sprouts in pixels, branching was defined by the number vessel branching points. Within each individual experiment the different the level of sprouting and branching was normalised to that of the untreated control samples to control for inter-assay variability.

1.2 Matrigel Assay

Growth factor reduced Matrigel (BD Biosciences) was defrosted on ice, whilst pipette tips and a flat-bottomed 96-well plate were chilled at -20 ^{o}C for at least 30 minutes. Whilst the 96-well plate and tips were kept on ice, 50 µL of Matrigel was added to the required number of wells. The plate was

then placed in an incubator at 37 ^{o}C for 30 minutes to allow the matrigel to set. Human umbilical vein endothelial cells (HUVEC) at passages 3-5 were used at a concentration of $2x10^4$ cells per well. HUVEC were re-suspended in EGM-2 (Lonza) containing either PBS (untreated control), +25 mM mannitol (control for osmotic stress) or +25 mM glucose. EGM-2 is a full growth media, containing FBS and growth factors, and this media was used for this experiment to investigate the impact of hyperglycaemia on EC tube formation, which would have been induced by FBS and other growth factors, and provides similar conditions to those used in the metatarsal assay. The assay was left for 18 hours to allow tube formation to occur, following this, the assay imaged by phase contrast microscopy. Tubule length and branching were quantified using Angiosys 1.0.

1.3 qPCR

RNA was extracted from cultured metatarsals or snap frozen mouse retinal tissue for quantitative PCR (qPCR) analysis, using the RNeasy Micro RNA kit (Qiagen), according to the manufacturers instructions. RNA amount, quality and purity were measured spectrophotometerically using a Nanodrop (Thermo Scientific). 1 µg of RNA was used to make cDNA using the quantitect reverse transcription kit (Qiagen) according to the manufacturers instructions.

Gene		Sequence (5'-3')
Ang2	Fwd	CAGCCACGGTCAACAACTC
	Rev	CTTCTTTACGGATAGCAACCGAG
Angptl2	Fwd	GAAGCCTGAGAATACCAACCG
	Rev	CCTTGCTTATAGGTCTCCCAGT
Angptl6 (exons 1-6)	Fwd	CCCGCAGAAGGCAACTAGC
	Rev	TCTTGGGTGGCTTCTGAGC
Angptl6 (exons 4-6)	Fwd	CCGTGTGGTGTGAACAGCAG
	Rev	TGAAGAAGTTGACAGAGCCGTC
Il6	Fwd	CTCTGGGAAATCGTGGAAAT
	Rev	CCAGTTTGGTAGCATCCATC
Lrg1	Fwd	CCATGTCAGTGTGCAGATTC
	Rev	AAGAGTGAGAGGTGGAAGAG
Plvap	Fwd	GCCAGGTGGTTGGACTATCTG
	Rev	CTCCATCTCACGTCGCGTA
Tgfbeta	Fwd	TTGCTTCAGCTCCACAGAGA
	Rev	TGGTTGTAGAGGGCAAGGAC
Vegf	Fwd	GACTTGTGTTGGGAGGAGGA
	Rev	TCTGGAAGTGAGCCAATGTG

Table 1: Primers used for qPCR analysis

Power SYBR green master mix (Life Technologies), primers (listed in Table 1, purchased from Invitrogen) diluted to 5 nM, cDNA and molecular biology grade water were combined and 20 µL of the reaction mixture was added per well. Each reaction was performed in triplicate and ran on a QuantStudio 6 Real Time PCR machine (initial denaturation: 95 ^{o}C 10 minutes, followed by 40 cycles of 15 s at 95 ^{o}C and 60 s at 60 ^{o}C) fluorescence was recorded at each cycle during the annealing step. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

1.4 ELISA

Mouse Vegf DuoSet enzyme linked immunosorbent assay (ELISA), Mouse Il-6 DuoSet ELISA and Mouse Ang-2 quantikine ELISA kits (R&D Systems) were used according to the manufacturers instructions. Before analysis, samples were centrifuged at 4 ^{o}C for 10 minutes to remove particulates and the supernatant was diluted 1:3 with 1% BSA (Millipore) in PBS. The plate was coated with 0.4 µg/mL of capture antibody overnight, blocked for 1 hour with 1% BSA in PBS prior to the addition of samples and standards. Appropriate biotinylated detection antibodies were used at 100 ng/mL for 2 hours, followed by addition of streptavidin-HRP diluted 1:40 for 20 minutes. Between the additions of each reagent, the plate was washed 3 times with 0.05% Tween-20 in PBS.

The concentration of the protein interest was determined colourimetrically by the addition of 50 µL of H_2O_2 and tetramethylbenzidine. The colourimetric reaction was stopped by addition of 2N H_2SO_4 and optical density was measured at 450 nm and corrected at 570 nm. Protein concentrations were determined using regression analysis of a standard curve created from the standards.

1.4 SDS PAGE and Western Blot

4x Laemilli buffer with 100 µM dithiothreitol (DTT) was added to protein lysates and boiled at 95 ^{o}C for 10 minutes. 10 µg of protein was loaded per well in a 4-12% Bis-Tris sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen) and proteins were separated by electrophoresis at 150 V for 2 hours. The separated protein was then transferred onto a nitrocellulose membrane at 12 V overnight. To check for

even transfer of protein onto the membrane, the membrane was incubated in 5% ponceu red solution for 5 minutes.

The membrane was then blocked in 5% BSA in tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were incubated with appropriate primary antibodies at 1:1000 (phospho NF-κB p65 or NF-κB p65, both antibodies were purchased from Cell Signal Technologies) overnight in 5% BSA in 0.1% TBST. Membranes were washed 3 times for 5 minutes in 0.1% TBST. Appropriate HRP-conjugated secondary antibodies were added at 1:1000 in 5% BSA in 0.1% TBST for 2 hours. Membranes were then washed for 3 times for 15 minutes in 0.1% TBST. Substrate solution was then to each side of the membrane for 1 minute. Excess substrate solution was removed and membranes were sealed in plastic film. Chemiluminescence was detected by exposing the membrane to an X-ray film (Biocare). The amount of protein was quantified by densinometry analysis in ImageJ.

2 *In vivo* Techniques

2.1 Experimental animals

C57Bl/6 mice (Harlan) were housed in individually ventilated cages (IVCs) with a standard 12-12 hour light/dark cycle with regulated temperature and humidity. They were fed a standard laboratory diet and water *ad libitum*. Diabetic mice had their cages changed daily, and fasting blood glucose and bodyweight levels were measured every two weeks following the induction of diabetes.

Leucine rich glycoprotein 1 (Lrg1) KO mice (Wang et al. 2013) were maintained in the same conditions as described above. The Lrg1 KO genotype was confirmed by a negative PCR result for the Lrg1 gene and a positive PCR result for the LacZ reporter gene.

Genotype		Sequence (5'-3')
Lrg1	Fwd	AGGTGAGCGCGCAATGGCTTCA
	Rev	CAGATCCAACATATCCAGCTGC
LacZ	Fwd	TCCTGGTGGGAGAGGACTC
	Rev	GTCTGTCCTAGCTTCCTCACTG

Table 2: primers used for genotyping Lrg1 KO mice

2.2 Streptozotocin induced diabetes

Male mice were randomly assigned to either control or diabetic groups. 7 days before the induction of diabetes, mice were fasted for 4 hours. Body weight and blood glucose were measured using a Freestyle Optimum blood glucose meter (Abbot Diabetes) in order to establish baseline bodyweight and blood glucose levels. Diabetes was induced by 5 daily consecutive

intraperitoneal (i.p.) injections of freshly prepared streptozotocin (Enzo Lifesciences) at 55 mg/kg in pH 4.5 citrate buffer. Control mice received the same injections containing citrate buffer alone.

2.3 Fundus Imaging

The Micron III (Phoenix Research Labs) was used to image the retina using brightfield microscopy. Mice were anaesthetised with ketamine at 1.5 mg/kg and dormitor at 0.25 mg/kg. Pupils were dilated with 1% tropicamide (Chauvins Pharmaceuticals). In addition to brightfield imaging of the fundus, the retinal vasculature was imaged using the green filter settings following a subcutaneous injection of 100 µL 1% fluorescein isothiocyanate (FITC) solution. Images were taken centred on the optic nerve. Immediately after image capture, anaesthesia was reversed with anti-sedan at 0.1 mg/kg.

2.4 Preparation of mouse retinal cryosections

Freshly enucleated eyes were fixed in 2% PFA in 2x PBS for 15 minutes. Following this excess tissue was trimmed from the posterior part of the eye and an incision was made anterior to the ora serata. The anterior part of the eye was cut away and removed along with the lens. The remaining eyecup was post-fixed in 4% PFA for 1 hour then added to a 30% sucrose solution overnight. The next day the eyecup was embedded in optimal cutting temperature compound (OCT) and rapidly frozen in dry ice cooled acetone before being stored at -80 ^{o}C. 10 µM sections were cut in a cryostat at -20 ^{o}C and captured on superfrost adhesion slides (VWR). Slides were allowed to dry at room temperature before being stored at -80 ^{o}C.

Sections were blocked for 1 hour at room temperature in 1% BSA with 0.5% Triton in PBS, followed by incubation with appropriate primary antibodies (Rabbit anti-ionised calcium-binding adapter molecule 1 (Iba1); 1:200, Rabbit anti-NF-κB; 1:100, Donkey anti-mouse IgG-488, 1:1000) in fresh blocking buffer at 4 ^{o}C overnight. Sections were washed three times with 0.1% Tween-20 in PBS for 5 minutes. All secondary antibodies were purchased from Invitrogen, raised in donkey and conjugated to alexa-fluor-488, -555 or -647. Appropriate secondary antibodies diluted 1:1000 in blocking buffer. Sections were then washed twice in 0.1% Tween-20 for 15 minutes before being counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 1:1000 in PBS for 10 minutes. Sections were then washed twice in PBS before being mounted under coverslips with DAKO fluorescent mounting medium (DAKO).

2.5 Preparation of mouse retinal flatmounts

Freshly enucleated eyes were fixed in 4% PFA for 1 hour. An incision was posterior to the ora serata, before being cut laterally with dissecting scissors to remove the anterior part of the eye. The optic nerve was then cut and the neurosensory retina was carefully separated from the RPE-choroid layer. Four radial incisions were made at 90^{o} to each other towards the centre to allow the retina to lay flat. The retina was then post-fixed in ice-cold methanol for 15 minutes. Retinas were either stored at -20 ^{o}C in methanol or the immunostaining protocol was started.

Prior to immunofluorescence staining, retinas were rehydrated with five washes in PBS. Retinas were then blocked for 1 hour at room temperature

in 0.5% BSA, 3% Triton and 1% Tween-20 in PBS. After blocking, retinas were incubated with the appropriate primary antibodies overnight at 4 ^{o}C in the blocking solution with gentle agitation. Retinas were then washed with fresh blocking solution three times for 15 minutes. Appropriate secondary antibodies were diluted 1:1000 in blocking buffer and added for 2 hours at room temperature. The retinas were then washed with fresh block three times for 30 minutes before being counterstained with DAPI for 10 minutes in PBS. After two further washes in PBS, retinas were mounted with DAKO fluorescent mounting medium (DAKO).

2.6 Intravenous administration of FITC-dextran

Mice were warmed in a 37 ^{o}C heat box to induce vasodilation before being restrained. 100 µL of 5 mg/mL of 40 kDa FITC-dextran (Invitrogen) was injected into the tail vein using a 30 G needle. The dextran was allowed to circulate for 1 hour before the mice were culled by cervical dislocation and tissue was extracted.

3 Statistics

Students t-test was used to analyse the difference between samples in all data. Welch's t-test was used to analyse the difference in samples from *in vivo* work due to the unequal variance in the two groups, due to a statistically significant difference following an F-test. All data are expressed as the mean with error bars as the standard error of the mean (SEM).

Chapter 3 Results

The link between hyperglycaemia and inflammatory-mediated vascular dysfunction in diabetes has been extensively studied *in vivo* (Bakker et al. 2009; Kolluru et al. 2012). Various factors have limited the effectiveness of equivalent *in vitro* studies, such as; differences between macrovascular and microvascular endothelial cells (Duffy et al. 2006), the lack of appropriate controls for osmotic stress (Yin et al. 2012; J. Zhao et al. 2015), and the relatively short-term nature of *in vitro* studies. Despite this, hyperglycaemia has been demonstrated to have some direct effect on endothelial cells *in* vitro, such as endothelial senescence (Maeda et al. 2015) and apoptosis (Sheu et al. 2005).

Various *in vitro* studies indicate that exposure of perivascular cells to high glucose alters the behaviour of endothelial cells via paracrine mediators (Kim 2002; Busik et al. 2008; Pfister et al. 2008; Yun et al. 2017). This indicates that high glucose-induced dysfunction in perivascular cells has an important role to play in diabetes-associated vascular dysfunction. Therefore, whilst treating endothelial cells with high glucose allows us to perform relatively simple studies on the effects of hyperglycaemia, utilising more complex, multicellular models offers greater insight into diabetes-induced changes to the vasculature. It is important to note that these *in vitro* models are still unable to fully recapitulate the diabetes-induced changes to the vasculature that occur *in vivo.* Since vascular changes in diabetic retinopathy occur in the context of the systemic vasculature, diabetes induced changes may in other organs may indirectly lead to changes in the retinal vasculature such as through circulating immune cells and endocrine signalling. Hence, the requirement for animal models of

diabetes, which will be discussed in the next chapter. An overview of the different models used in this thesis (Figure 8).

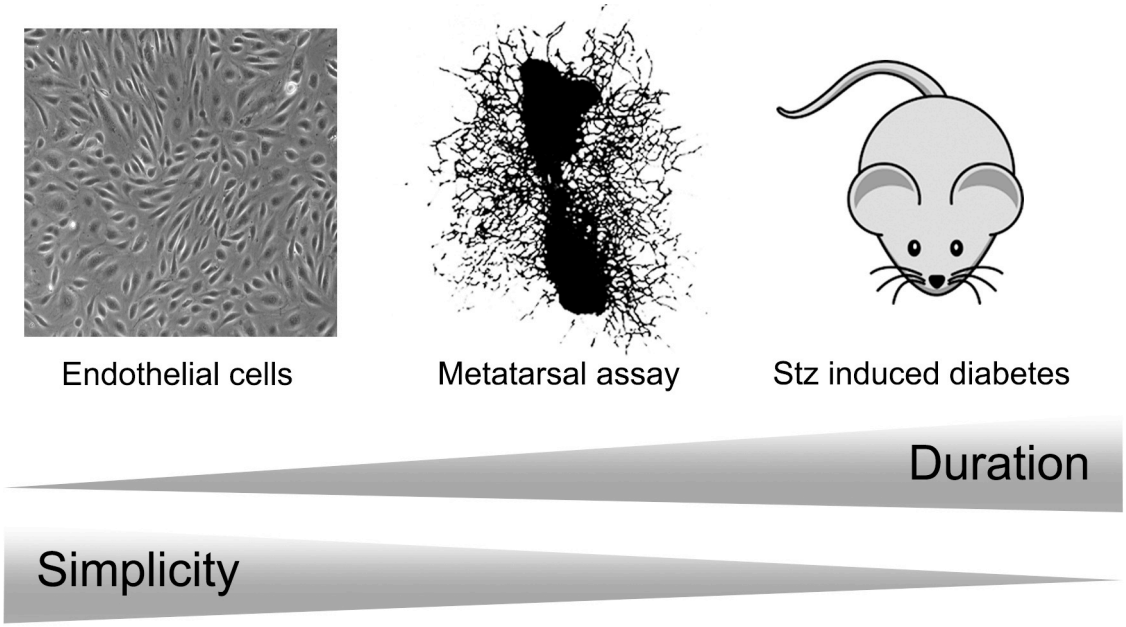

Figure 8: Schematic of the different experimental models used in this thesis. The three models, in order of increasing complexity are cultured endothelial cells, the mouse embryonic metatarsal assay and the streptozotocin (Stz) induced diabetic mouse, an *in vivo* model of type 1 diabetes.

1 Hyperglycaemia and angiogenesis *in vitro*

To investigate the role of hyperglycaemia on endothelial cell proliferation and tube formation, the Matrigel assay was used. HUVEC were seeded onto Matrigel-coated wells of a 96-well plate in culture media containing PBS, 25 mM mannitol or 25 mM glucose. Glucose concentrations ranging between 20 and 30 mM have been widely used to replicate hyperglycaemia *in vitro* (Duffy et al. 2006; Sheu et al. 2005; Suarez et al. 2015; X.-Y. Zhao et al. 2015). Additionally, this concentration matches the blood glucose level frequently observed in mouse models of diabetes (Figure 28). The assay

was performed for 18 hours overnight before tube formation was imaged and quantified. Compared to PBS (Figure 9 A) and mannitol (Figure 9 B), tube formation was significantly ($p<0.001$) reduced by 30% in the high glucose treatment (Figure 9 C), although there was no quantitative change in the amount of branching (Figure 9 E).

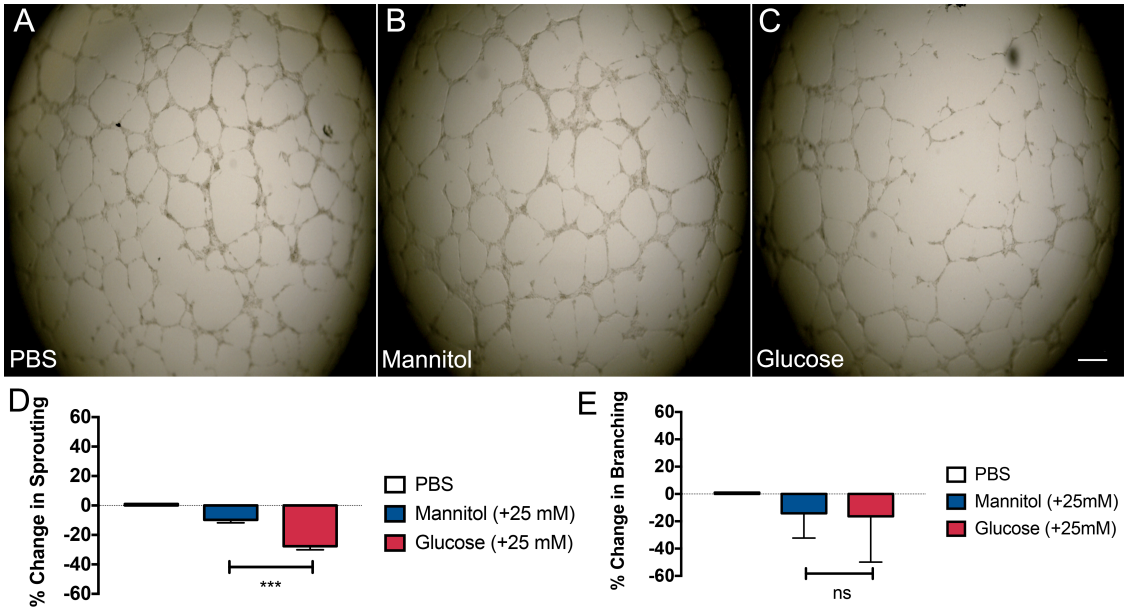

Figure 9: High glucose reduces tube formation in the Matrigel assay. Representative images taken by phase contrast of the HUVEC Matrigel assay with PBS (A), 25 mM mannitol (B) or 25 mM glucose (C). Quantification of sprouting length showed a reduction in glucose-treated samples compared to PBS or mannitol-treated assays (D). No change in branching was observed between treatments (E). Data representative of 3 independent experiments with at least 6 wells used for each treatment in each experiment. Stastical analysis performed using studnets T-test. *** = $p<0.001$. Scale bar = 500 µm.

Having shown that tube formation in the Matrigel assay was impaired in hyperglycaemic conditions, the effects of hyperglycaemia on angiogenesis in the metatarsal assay were investigated. The metatarsal assay is a multicellular angiogenesis involving endothelial cells with associated mural cells such as pericytes, fibroblasts and macrophages, and doesn't require the addition of exogenous growth factors (Deckers et al. 2001; Song et al. 2015). This permits the investigation of effects of hyperglycaemia on angiogenesis in a multicellular environment. To our knowledge, this is the first time the effects of hyperglycaemia have been investigated in a multicellular model of angiogenesis.

Three days after dissecting and plating the metatarsals, the cultures had attached and established with vessel sprouts starting to form. The media was changed and replaced with treatments of either PBS, 25 mM mannitol (the osmotic control) or 25 mM glucose. Media containing the relevant treatments was changed four times every two days. Compared to PBS and mannitol treatments (Figure 10 A-B), metatarsals cultured in media with 25 mM glucose (Figure 10 C) had a 25% reduction ($p<0.05$) in vessel sprouting and branching (Figure 10 D-E). This indicates that elevated glucose levels impair new vessel growth, as observed in the Matrigel assay (Figure 9) and that osmotic stress is unlikely to have played a role in reducing the total level of sprouting.

Taken together, Figure 9 and Figure 10 show that hyperglycaemia impairs the formation of vessel sprouting, and that these relatively simple models

may re-capitulate aspects of diabetes-induced vascular dysfunction which result from elevated blood glucose.

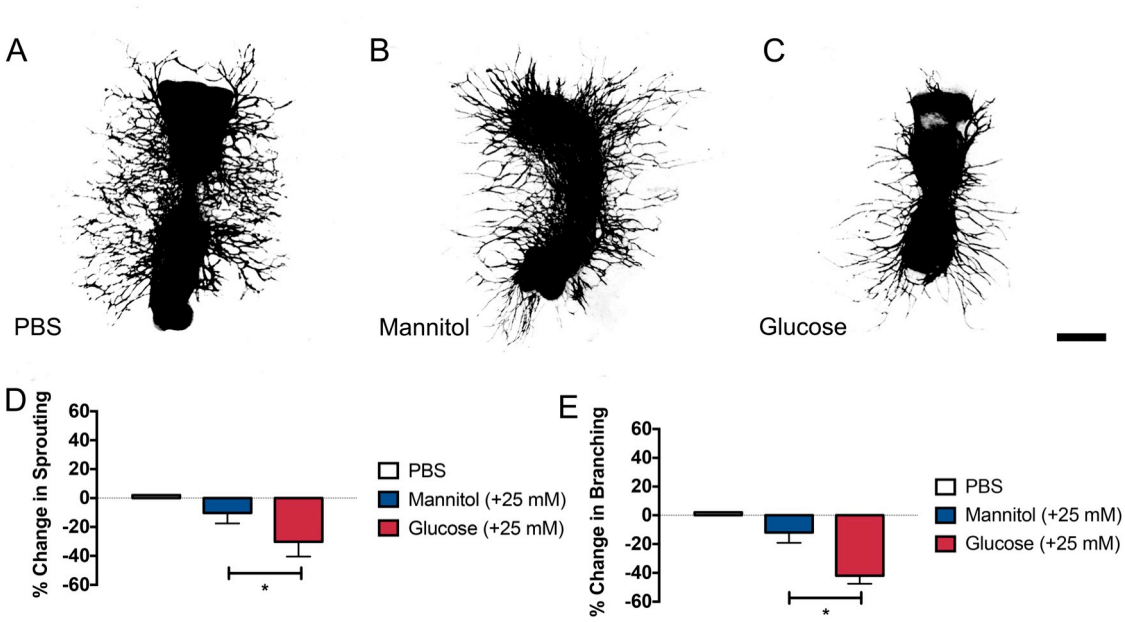

Figure 10: High glucose reduces vessel growth and branching in the metatarsal assay. Metatarsals were cultured for 10 days with PBS, 25 mM mannitol and 25 mM glucose, and then fixed and stained for CD31 to label endothelial cells (A-C). Quantitative analysis of CD31 staining to measure vessel sprouting (D) and vessel branching (E) showed that high glucose reduces vessel sprouting compared to PBS or mannitol controls. Data from 4 independent experiments with at least 12 individual bones used for each treatment in each experiment. Statistical analysis was performed using students T-test. * = $p<0.05$. Scale bar = 500 µm.

Altered expression of pro-angiogenic factors such as *Vegf* and *Ang2* has been shown to promote vascular dysfunction in diabetic retinopathy (Ishida et al. 2003; Yun et al. 2016). As expression of these and other angiogenic factors may be altered by hyperglycaemia, I next investigated whether reduced vessel sprouting in the metatarsal assay (Figure 11) was accompanied by changes in growth factor expression. Whilst a more exhaustive approach would be to perform microarray to investigate changes to the transcriptome and proteome in response to high glucose, the costs associated with this technique meant that a candidate gene based approach was utilised instead.

Because Vegf plays an important role in angiogenesis, both gene expression and protein secretion were examined (Figure 11 A-B). qPCR analysis showed that there was no significant difference in the level of mRNA expression in high glucose conditions compared to controls (Figure 11 A). In contrast, the level of Vegf detected in the conditioned media was reduced in high glucose treated metatarsals compared to controls (Figure 11 B), consistent with the data in Figure 9 and Figure 10. Since Ang-2 also plays a key role in promoting angiogenesis (Felcht et al. 2012), we measured its mRNA and protein levels. Similar to *Vegf*, no significant change in the level of *Ang2* mRNA was detected (Figure 11 C), however there was a small, ~20% but non-significant reduction in the level of Ang-2 in the conditioned media. Lrg1 has been previously been shown to be upregulated in the vitreous of patients with PDR, and promotes angiogenesis and vascular leakage by modifying Tgf-β signalling (Wang et al. 2013). To investigate whether high glucose had any effect on Lrg1 and Tgf-β, qPCR analysis of

both genes was performed. No statistically significant changes in gene expression were observed (Figure 11 E-F). Analysis of these angiogenic factors suggests a trend towards a decrease in expression in the presence of high glucose, although this was not statistically significant. However, this trend does fit in with the previously described trend for a reduction in angiogenesis in response to high glucose treatment. The lack of statistical significance in the findings in Figure 11 indicate that the response to high glucose is relatively minor and that further work to optimise this assay in order to detect an enhanced response may reveal more robust changes in gene expression alongside other findings.

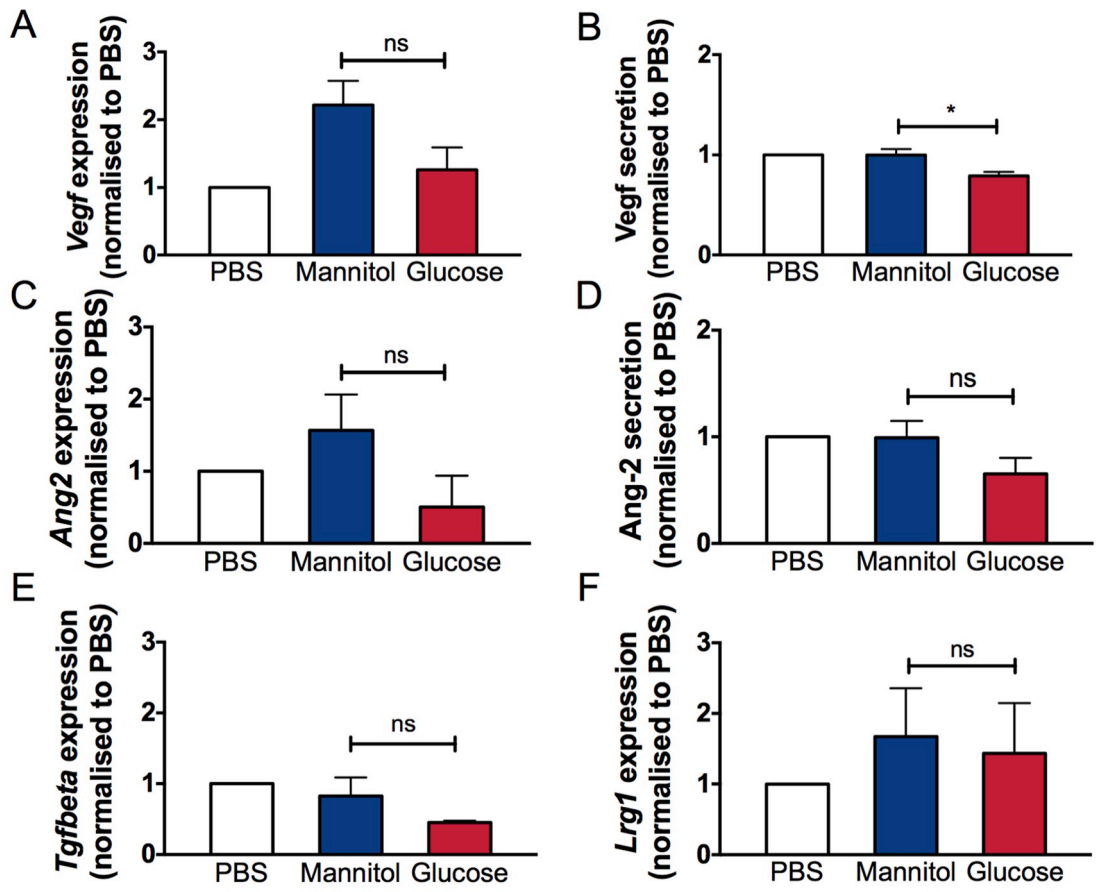

Figure 11: High glucose reduces Vegf secretion in the metatarsal assay, but does not alter the expression of other growth factors. Metatarsals were cultured with PBS, 25 mM mannitol or 25 mM glucose as described in Figure 9. RNA was extracted and prepared for qPCR analysis. ELISAs were performed on conditioned media taken the same day as the RNA extraction. Whilst *Vegf* expression was not altered (A), there was a small but statistically significant reduction in secreted Vegf in the conditioned media (B). *Ang-2* expression was not altered (C), but there was a slight but non-significant reduction in secreted Ang-2 in the conditioned media (D). There were no changes to *Tgfbeta* (E) or *Lrg1* (F) expression. Data are from 3 independent experiments with at least 12 individual bones per experiment. Statistical analysis was performed using students T-test. * = $p<0.05$.

Reduced pericyte coverage is known to be associated with vascular dysfunction in diabetic retinopathy (Ogura et al. 2017). High glucose has been shown to affect pericytes *in vitro*, by reducing contractility and proliferation, and increasing apoptosis (Beltramo et al. 2006; Suarez et al. 2015). Therefore, we were interested in seeing whether high glucose would alter the biology of pericytes and their association with endothelial cells in the metatarsal assay. Selecting appropriate markers for pericytes is challenging and can be contentious, as there is no universal marker for all pericytes, the expression of different markers changes as pericytes migrate and attach to the endothelium (Armulik et al. 2011), additionally the reliability of some commercially available antibodies cannot be guaranteed. Two of the most widely used markers for pericytes are α-SMA and NG-2. To determine which was most appropriate in the metatarsal assay, the staining of both markers was compared by immunofluorescence (Figure 12 A-C). NG2 (Figure 12 A) appeared to be a better marker than α-SMA (Figure 9 B) as its expression was restricted to perivascular cells (Figure 12 A-C), whilst α-SMA also appeared to be expressed in other cell types.

To assess the ratio of pericytes to endothelial cells, metatarsals were treated with PBS (Figure 12 D), 25 mM mannitol (Figure 9 E) or 25 mM glucose (Figure 12 F) as previously described. After 10 days in culture, the metatarsals were fixed and stained for CD31 and NG2, and the ratio of NG2 to CD31 staining was quantified (Figure 12 G). No change in the ratio of NG2 to CD31 was detected which implies that there were no alterations to pericyte staining upon high glucose treatment.

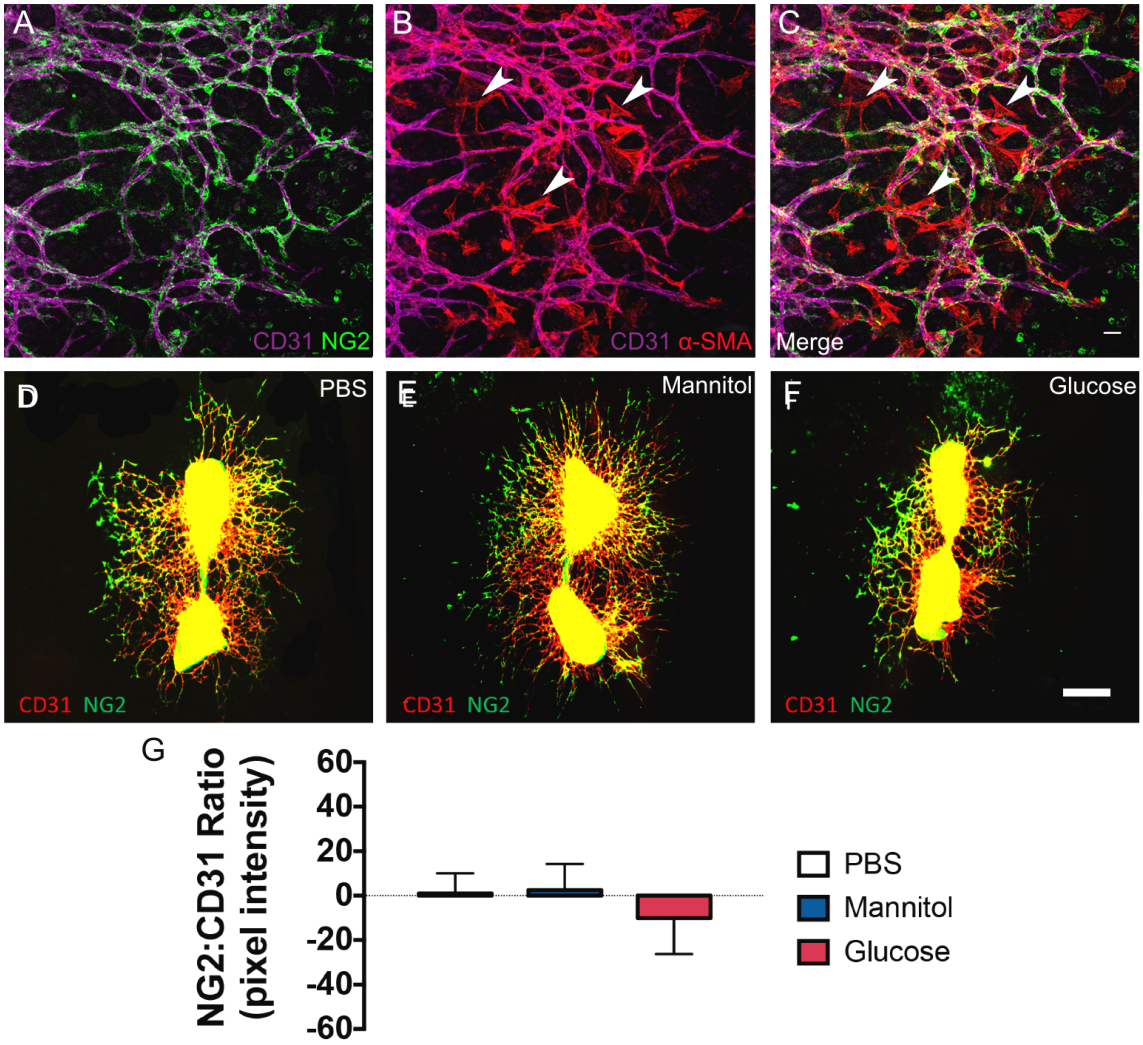

Figure 12: High glucose does not alter the pericyte-endothelial cell ratio in the metatarsal assay. Microscopy was used to identify a suitable pericyte marker to quantify pericyte coverage in the metatarsal assay (A-C). Staining for CD31 in combination with NG2 (A) and α-SMA (B) showed that NG2 is a more appropriate marker of pericytes in this assay. Whilst NG2 and α-SMA showed some co-localisation (C), α-SMA also stained cells that were not directly associated with endothelial cells (white arrowheads). Representative low power images of CD31 and NG2 staining in metatarsals treated with PBS (D), 25 mM mannitol (D) or 25 mM glucose (E) as previously described in Figure 10. The image area containing the bone was masked, and the ratio of NG2 to CD31 staining was quantified (G) indicating there is no change to pericyte staining in metatarsals cultured under high glucose conditions. Data representative of two independent experiments. Scale bar = 10 μm (A-C), 500 μM (D-F).

2 AGE-BSA and angiogenesis *in vitro*

Supplementing growth media with glucose is a simple strategy to investigate the effects of hyperglycaemia *in vitro*, however, it fails to replicate the complex nature of the diabetic environment *in vivo*. An increase in advanced glycation end products (AGEs), such as circulating glycated haemoglobin, have been linked to the incidence and severity of microvascular complications of diabetes such as DR (Sabanayagam et al. 2009). AGE measurements therefore provide a better indicator of glucose control and diabetes severity than fasted blood glucose measurements. Additionally, AGE formation has also been linked to the pathophysiology of diabetic retinopathy in mouse models of experimental diabetes (Stitt 2010). AGE induces inflammation primarily through activation of its receptor, RAGE, and its downstream pro-inflammatory signalling pathways, as well as through other non-RAGE signalling pathways (Stirban et al. 2014). Therefore, in addition to acting as a biomarker for diabetes severity, circulating glycated proteins may contribute to diabetes-induced vascular dysfunction. RAGE blockade, with a soluble RAGE isoform improved wound healing (Goova et al. 2001), and treatment with a RAGE-IgG fusion protein reduced the severity of diabetic retinopathy (Li et al. 2011), both in diabetic mice. These studies therefore indicate a direct role for AGE/RAGE signalling in mediating the vascular complications of diabetes.

Albumin is an abundant circulating protein, and can undergo modification with AGEs *in vivo* so this constitutes a suitable reagent for the *in vitro* delivery of AGEs. Therefore, in an attempt to better model the effects of

diabetes on the vasculature, metatarsals were cultured with exogenously added AGE-BSA to investigate the effects of AGE in this explant model.

Untreated (Figure 13 A), 100 µg/mL BSA (Figure 13 B) or 100 µg/mL AGE-BSA (Figure 13 C) treatments were added on the third day in culture and replaced every two days. Quantification of vessel sprouting (Figure 13 D) and branching (Figure 13 E) showed that AGE-BSA (Figure 13 C) lead to a two-fold increase ($p<0.05$) in sprouting of new vessels compared to untreated and BSA treated metatarsals (Figure 13 A-B), in contrast to our previous findings with high glucose treatments (Figure 10). This could be explained by the different mechanisms of action of high glucose and AGE-BSA on the vasculature when they are given separately. In an *in vivo* model of diabetes both hyperglycaemia, AGE-BSA and other factors would no doubt act in concert to provide the diabetic phenotype.

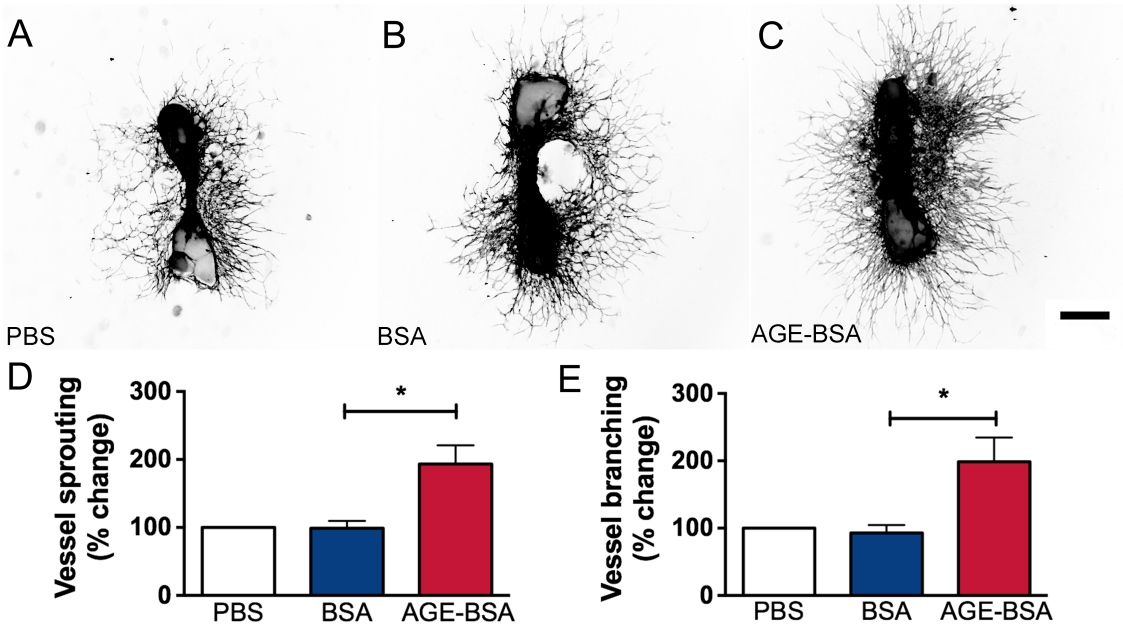

Figure 13: AGE-BSA increases vessel growth and branching in the metatarsal assay. Representative images of metatarsals cultured as previously described with growth media supplemented with; PBS (A), 100 µg/mL BSA (B) or 100 µg/mL AGE-BSA (C). Quantitative image analysis of CD31 staining was performed to measure vessel sprouting (D) and branching (E) which revealed that 100 µg/mL AGE-BSA increased both vessel sprouting and branching. Data are from 3 independent experiments with at least 12 bones used per treatment in each experiment. Statistical analysis was performed using students T-test. * = $p<0.05$. Scale bar = 500 µm.

AGE-BSA induced a two-fold increase in vessel sprouting (Figure 13), and AGE-BSA has been previously shown to alter gene expression *in vitro* (Wang et al. 2011), including that of VEGF (Yamagishi et al. 1997), probably through NF-κB dependent signalling (Hoesel & Schmid 2013). I therefore decided to investigate whether AGE-BSA treatment upregulated Vegf and the expression of other angiogenic genes in the metatarsal assay. After the final treatment, the RNA was extracted and prepared for qPCR analysis. AGE-BSA treatment led to a three-fold ($p>0.05$) increase in both Vegf mRNA (Figure 14 A) and protein (Figure 14 B) levels, which is in agreement with previous *in* vitro studies showing that AGE-BSA induces VEGF production (Yamagishi et al. 1997).

As Lrg1 and Tgf-β work together to induce angiogenesis, the expression of both genes was analysed by qPCR to determine whether their expression was induced by AGE-BSA treatment. Whilst *Tgfbeta* (Figure 14 D) and *Lrg1* (Figure 14 C) expression appeared to be increased two-fold and four-fold respectively in response to AGE-BSA, only the increase in *Lrg1* was statistically significant. Since Il-6 has been shown to be increased in diabetic retinopathy (Chen et al. 2016) and pathological angiogenesis (Gopinathan et al. 2015), Il-6 mRNA (Figure 14 E) and protein (Figure 14 F) were also measured. *Il6* mRNA was markedly upregulated with a 60-fold increase in response to AGE-BSA treatment, in contrast to its low level of expression under control conditions. A similar pattern for the 60-fold increase in Il-6 protein levels was also observed (Figure 14 F), however, since the data are from only two independent experiments statistical analysis was not performed.

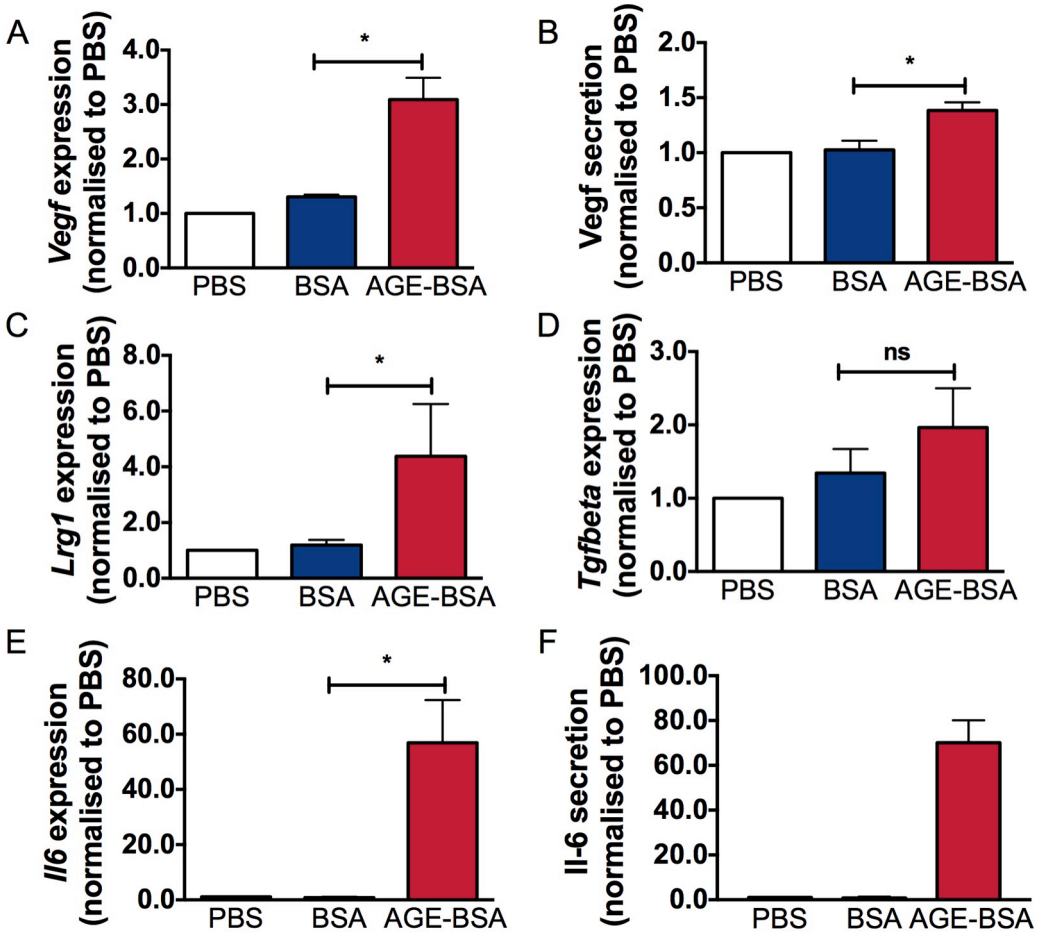

Figure 14: AGE-BSA increases the production of pro-angiogenic and pro-inflammatory growth factors in the metatarsal assay. Metatarsals were cultured as described in Figure 10. RNA was extracted and prepared for qPCR analysis, gene expression was normalised to the endogenous control, *Hprt*. ELISAs were performed on conditioned media taken the same day as the RNA extraction. Vegf mRNA (A) and protein (B) levels were increased following treatment with 100 µg/mL AGE-BSA compared to control treatments. *Lrg1* (C) and *Tgfbeta* (D) expression appeared to be increased in response to AGE-BSA treatment, however, this increase was not statistically significant. In contrast, *Il6* expression was strongly induced in response to AGE-BSA treatment (E). A similarly strong increase in secreted Il-6 was also detected but statistical analysis was not performed as there were only two experimental replicates. Data are from 3 independent experiments, apart from F, which is the result of 2 independent experiments. Statistical analysis was performed using students T-test. * = $p<0.05$.

Having shown a 60-fold increase in Il-6 mRNA and protein in response to AGE-BSA treatment (Figure 14 E-F), which in agreement with (Figure 13) implies that AGE-BSA exerts a pro-angiogenic effect in the metatarsal assay. I was interested in what effect the increase in Il-6 would have on the metatarsal assay. The janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is activated by IL-6, which culminates in phosphorylation of the STAT3 transcription factor, and has a well-established role in inflammation and angiogenesis (Scheller et al. 2011; Oh et al. 2012; Yun et al. 2017). In order to determine whether the increase in Il-6 also increased the level of JAK/STAT signalling, cultured metatarsals were treated with 100 µg/mL BSA or 100 µg/mL AGE-BSA and stained for pSTAT3 (Figure 15).

Although not statistically significant, staining for pSTAT3 was increased by 10% in AGE-BSA treated metatarsals (Figure 15 A-C), compared to control metatarsals, which were treated with BSA alone (Figure 15 D-F). Quantification of pSTAT3 staining confirmed a trend towards an increase in pSTAT3 staining in AGE-BSA treated samples (Figure 15 E) However, caution must be used when interpreting this finding, as the pSTAT3 staining was not specific to the nucleus and this experiment was only performed once with only three samples per treatment. Nevertheless, the increase in STAT3 phosphorylation demonstrated here suggests that one mechanism through which AGE-BSA increases angiogenesis may be through Il-6 dependent activation of STAT3, mediating increased angiogenesis in a pro-inflammatory dependent context.

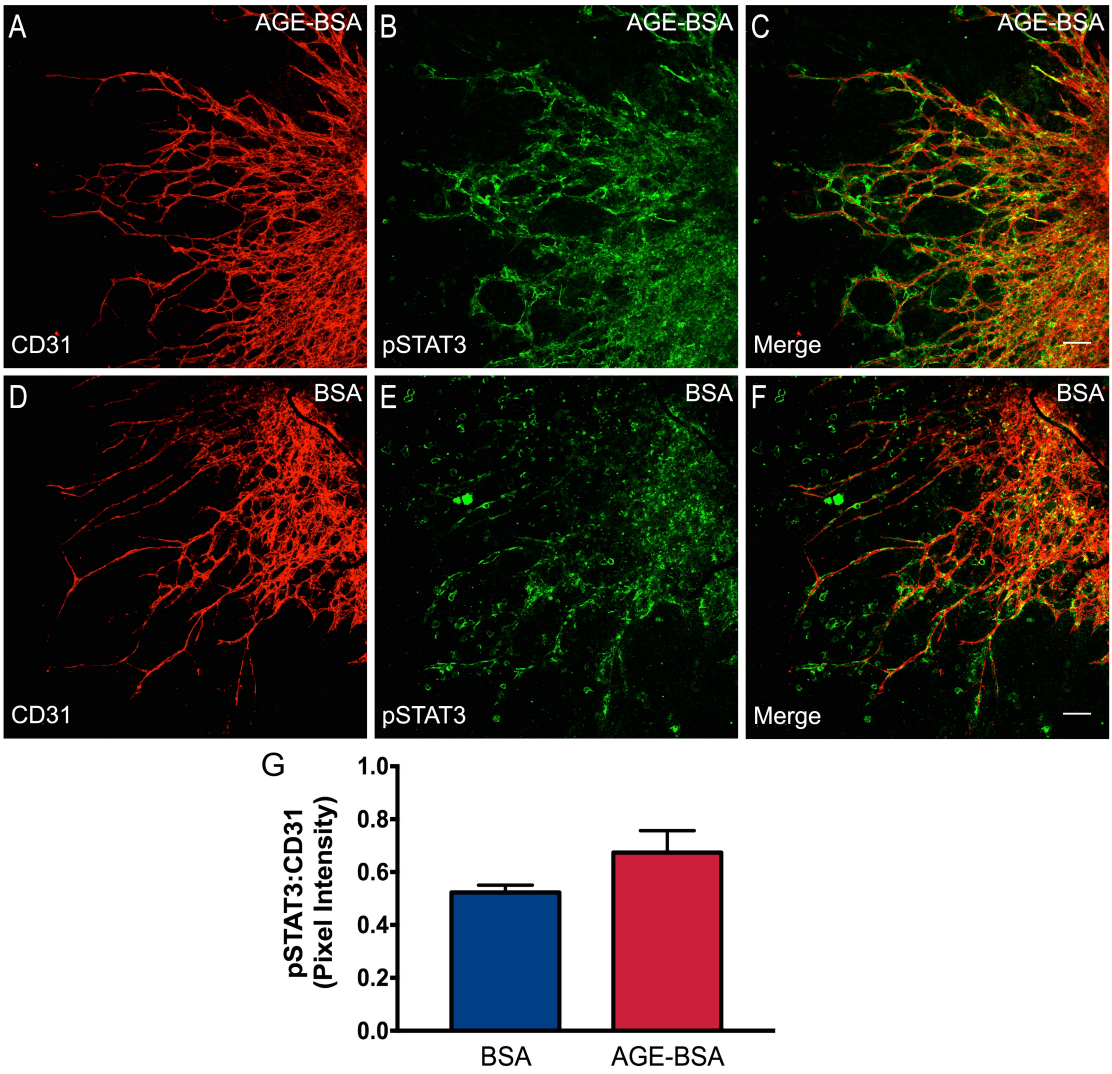

Figure 12: Staining for pSTAT3 is increased in AGE-BSA treated metatarsals. Metatarsals were cultured as previously described with either 100 µg/mL BSA or 100 µg/mL AGE-BSA. Samples were stained for CD31 to label endothelial cells, and a phospho-specific antibody to STAT3 (pSTAT3) to examine STAT3 phosphorylation. The level of pSTAT3 appears to be increased in AGE-BSA treated (A-C) over BSA treated (D-F) metatarsals. pSTAT3 staining was quantified and normalised to CD31 staining (G) which revealed a trend for increased pSTAT3 staining in AGE-BSA treated samples. Data from 3 bones per treatment and representative of one independent experiment. Scale bar = 100 µm.

Plasmalemma vesicle associated protein (Plvap) is an endothelial cell-specific marker of increased vascular permeability and is expressed at fenestrae and caveolae. Whilst these organelles are unlikely to have a functional role in the vascular network produced in the metatarsal assay, both play an important role in mediating vascular permeability amongst other functions *in vivo*. Additionally, elevated levels of Plvap are associated with disease-induced vascular abnormalities (Guo et al. 2016), and have been reported to be associated with vascular leakage in the Akimba mouse model of diabetes (Wisniewska-Kruk et al. 2014). Plvap upregulation is also induced by Vegf (Wisniewska-Kruk et al. 2016). Considering the previous data showing AGE-BSA induced angiogenesis (Figure 13) and Vegf upregulation (Figure 14), I next asked whether Plvap is also up-regulated in response to AGE-BSA treatment.

To determine whether AGE-BSA altered the level of Plvap, qPCR and IF analysis were performed on cultured metatarsals. Following AGE-BSA or BSA (Figure 16 A-F) treatment, the intensity of Plvap staining was quantified and normalised to Collagen IV staining (Figure 16 G). However, only a very slight increase in Plvap staining was detected suggesting that AGE-BSA has little impact on Plvap expression in the metatarsal assay. This observation was confirmed by qPCR analysis of *Plvap* mRNA (Figure 16 H) where no significant change in *Plvap* expression was observed.

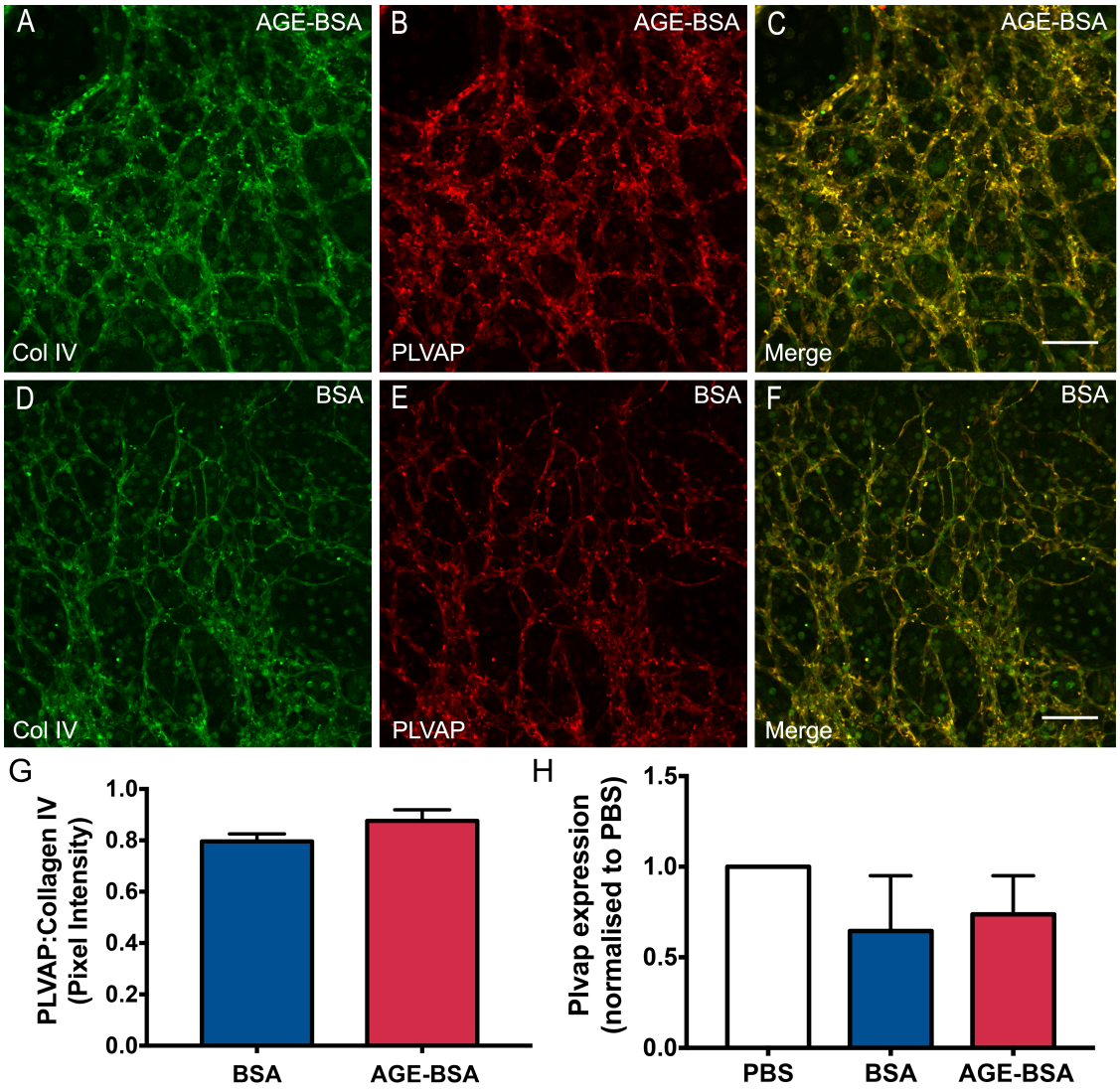

Figure 16: Plvap is not altered by AGE-BSA treatment in the metatarsal assay. Metatarsals were cultured with 100 µg/mL AGE-BSA (A-C) or 100 µg/mL BSA (D-F) as previously described. Plvap staining was quantified and normalised to collagen IV staining (G) and there was only a slight increase in Plvap staining intensity in AGE-BSA treated metatarsals. qPCR analysis of *Plvap* mRNA showed there was no alteration in the level of gene expression (H). Scale bar = 100 µm. Data representative of two independent experiments.

After completing the experiments, and because of insufficiently detailed information from the manufacturer (Millipore) about the purity of the AGE-BSA, I performed an endotoxin test on the material. This is because endotoxin has previously been shown to exert a pro-angiogenic effect on endothelial cells and also activates inflammatory signalling pathways, which are downstream of RAGE (Pollet et al. 2003).

Although testing revealed the presence of endotoxin, it is not clear whether contamination occurred during the course of the study, or, if earlier batches of this reagent were affected, and time did not permit an investigation of this issue. Testing did reveal that the endotoxin concentration when the AGE-BSA was diluted to its working concentration of 100 µg/mL would have been ~ 10 pg/mL. Other studies have shown endotoxin to induce angiogenesis at concentrations >2 ng/mL *in vivo* (Mattsby-Baltzer et al. 1994) and >100 ng/mL *in vitro* (Pollet et al. 2003; Li et al. 2017). Other reports have shown AGE-BSA to induce VEGF expression (Mamputu & Renier 2004), in which the authors explicitly state in the manuscript that endotoxin testing was performed on their AGE-BSA preparations. To determine what concentration of endotoxin affected angiogenesis in the metatarsal assay exogenous endotoxin could be titrated in to determine the minimum concentration of endotoxin required to induced angiogenesis in this model. Repeat studies using AGE-BSA confirmed to be endotoxin-free would need to be performed before drawing too many conclusions from these data.

Chapter 4 Results

Whilst *in vitro* and *ex vivo* models are useful tools for vascular biology research, they are not able to replicate the complex nature of the vasculature in living tissue. Additionally, the unique biology and complex neurovascular interactions in the retina differ substantially from what can be replicated *in vitro*. Therefore, to gain an understanding of a complex vascular complication of diabetes such as diabetic retinopathy, animal models of diabetes are essential.

The streptozotocin-induced mouse model of diabetes is widely used to study diabetes *in vivo*, with a Pubmed search for 'streptozotocin mice' returning over 6,000 results. Streptozotocin enters β-cells through glucose transporter 2 (Glut-2) and exerts its cytotoxic effect through DNA damage-mediated activation of the Poly-ADP ribose polymerase (PARP) apoptosis pathway (Chaudhry et al. 2013). β-cells are responsible for insulin production, and their destruction leads to hyperglycaemia due to a lack of insulin production. Other widely used models of diabetes to study diabetic retinopathy include; Ins2Akita and Akimba mice as well the streptozotocin induced diabetic rat. The main advantage of these other models is that they have been reported to recreate the severity and the range of features of human diabetic retinopathy in a shorter time frame. However, the initial costs to acquire these models and the time and the animal usage required to cross these animals onto other transgenic mouse lines, and in the case with rats increased maintenance costs, means that the streptozotocin mouse model has certain practical advantages.

1 The streptozotocin-induced diabetic mouse retina

Therefore, the aim of work in this chapter was to examine the streptozotocin-induced diabetic mouse for signs of changes associated with diabetic retinopathy. One of the main aims of this study was to identify changes that occur prior to the onset of more overt pathology, and to try and identify novel targets for therapeutic intervention. There is at present a largely unmet clinical need for therapeutics to manage the early stages of DR, with the anti-VEGF Aflibercept being the frontline treatment, and effective in <50% of patients. Diabetes was induced with 5 daily i.p. injections of streptozotocin, with age-matched controls receiving i.p. injections of vehicle. Induction of diabetes was confirmed by at least 3 consecutive fasted blood glucose measurements >20 mM.

Fundus photography and fluorescein angiography are widely used in the clinic to non-invasively monitor diabetic patients for signs of retinopathy (Stitt et al. 2015) (Figure 4). The retinas of control and diabetic mice were initially examined by fundus imaging and fluorescein angiography (Figure 17) to assess any changes to the retina two months after diabetes induction. Unexpectedly, fluorescein angiography did not reveal any evidence of vascular leakage in diabetic compared to control (Figure 17 C-D) mice. However, analysis by fundus imaging suggested that there were some changes, with white spots observed in diabetic retinas (Figure 17 B, white arrowheads), which were absent in control (Figure 17 A) mice. Whilst there is no evidence of a severe phenotype in the retina from this analysis, this could indicate the presence of inflammatory infiltrates into the retina.

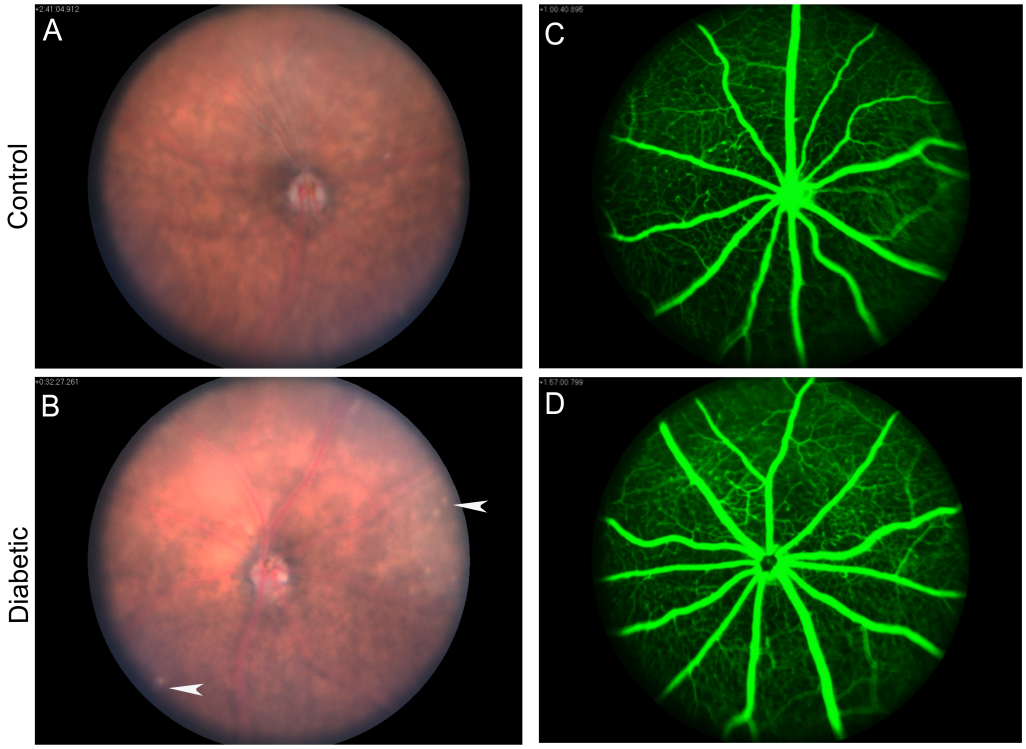

Figure 17: Fundus imaging and fluorescein angiography of diabetic and control mouse retinas. Fundus imaging was performed two months following diabetes induction. Brightfield imaging in control (A) and diabetic (B) retinas revealed some minor changes to the retina in diabetic mice (white arrowheads) which may indicate areas of inflammation. There was no evidence of vascular leakage in control (C) or diabetic (D) retinas analysed by fluorescein angiography imaged 10 minutes after subcutaneous injection of fluorescein. Images representative of 6 mice per condition.

Glial fibrillary acidic protein (GFAP) is a specialised intermediate filament protein expressed in astrocytes and Müller cells in the retina. GFAP expression is altered in diabetes and is a well-characterised sign of retinal stress (Feit-Leichman et al. 2005). GFAP expression in the Müller cell is a sign of gliosis, which contributes, to inflammation and vascular leakage (Bringmann & Wiedemann 2012). To investigate changes to the macroglia in the diabetic retina, GFAP expression was analysed in retinal cryosections two months after the induction of diabetes. In control (Figure 18 A-C) mice, GFAP expression was strictly limited to the ganglion cell layer (GCL). However, in diabetic mice (Figure 18 D-F), there was some indication of GFAP expression protruding deeper into the retina in what may be a terminal process of the Müller cell (Figure 18 D-F, white arrowhead). This suggests some evidence of mild stress in the retina. Since this is a minor phenotype which in itself is not indicative of functional change in the diabetic retina quantitative image analysis was not performed.

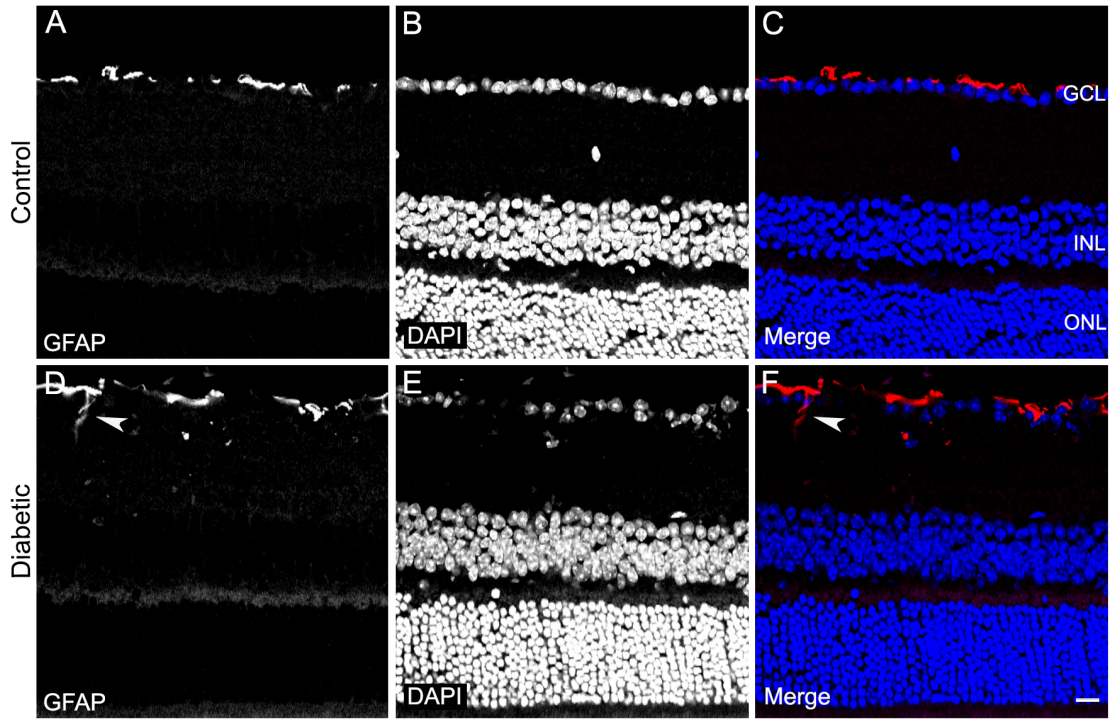

Figure 18: GFAP expression in control and diabetic mouse retinal cryosections. 12 µm cryosections were cut from OCT embedded mouse eyes and stained for GFAP to examine changes to the glial cells of the retina in diabetic mice two months after the induction of diabetes. In control mice (A-C) GFAP staining was strictly limited to the ganglion cell layer. In diabetic mice (D-F) some GFAP staining was seen in the inner plexiform layer (white arrowhead), but did not protrude deeper into the retina. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Images representative of sections taken from 3 different control and diabetic mice. Scale bar = 20 µm.

Microglia are a tissue resident population of macrophages, which normally reside in the inner plexiform layer (located between the GCL and INL). During retinal stress or inflammation they adopt an amoeboid and migratory phenotype and enter the ganglion cell layer and inner nuclear layer (Tang & Kern 2011). Since microglia are an important source of inflammatory mediators and play a role in the death of retinal neurons (Krady et al. 2005), microglial activation is a key step in the progression of diabetic retinopathy.

Mouse retinal cryosections were stained for Iba1 to investigate changes to the distribution and morphology of immune cells in the retina. Iba1 is a marker expressed in both the resident microglia of the retina and in infiltrating macrophages from the circulation in response to pro-inflammatory signalling. It is not possible to differentiate between resident microglia and recruited monocytes by IF (Caicedo et al. 2005), so Iba1 staining will label both tissue resident and recruited macrophages. $Iba1^{+}$ cells appeared to adopt an amoeboid phenotype and to have migrated into the GCL and the INL in sections from diabetic mice (Figure 19 D-F, white arrow heads), with neither feature apparent in control mice (Figure 19 A-C) Quantitative image analysis revealed that the number of infiltrating immune cells was significantly ($p>0.01$) increased in diabetic retinas compared to controls.

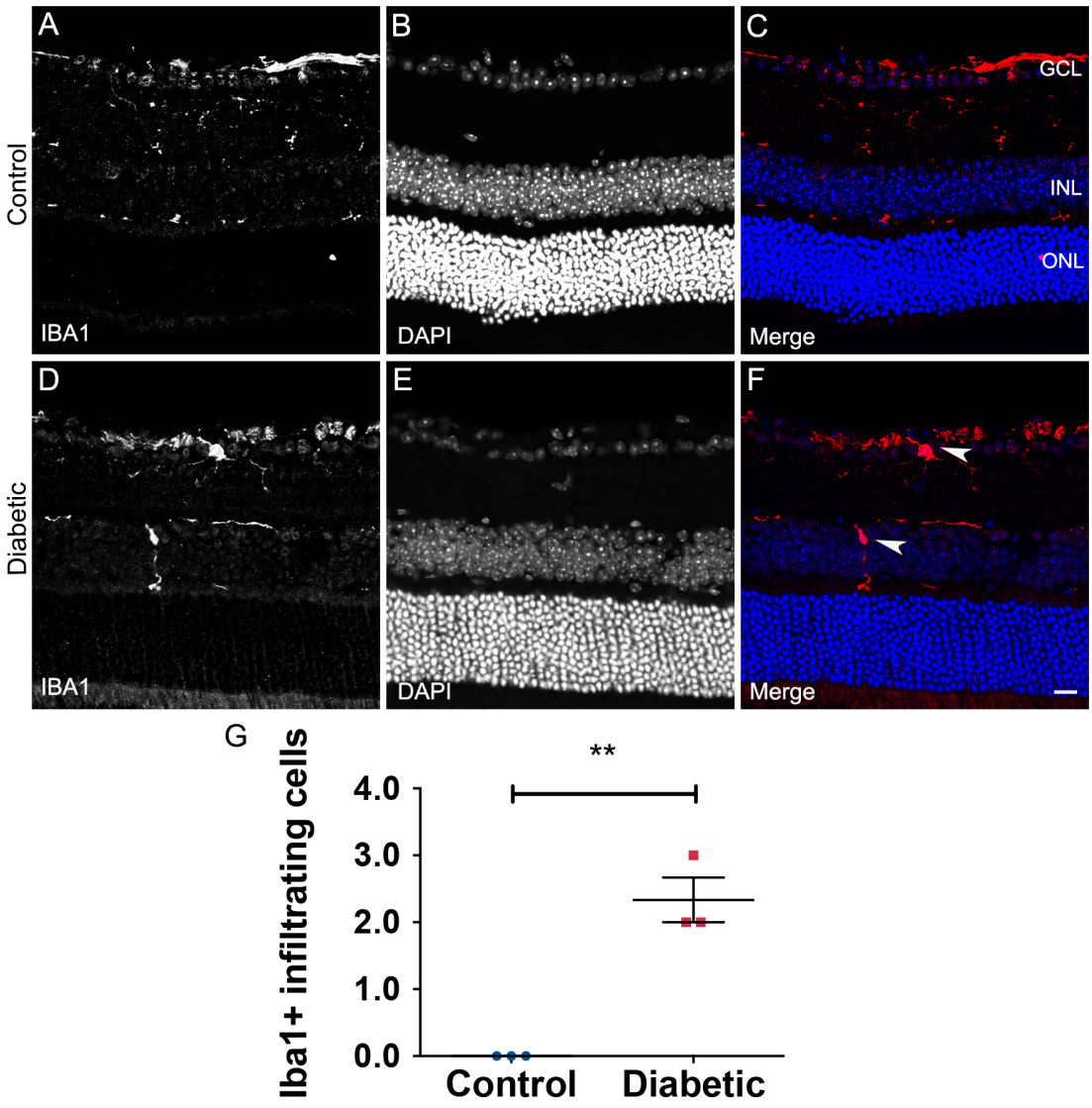

Figure 19: Iba1 staining in control and diabetic mouse retinal cryosections. 12 µm cryosections were stained for Iba1 to investigate changes to resident and infiltrating immune cells two months following the induction of diabetes. In control (A-C) retinas, a low level of Iba1 staining is found throughout the inner retina. In (A, C) there appears to be some non-specific staining in the ganglion cell layer. In diabetic (D-F) mice some microglia have undergone a conformational change and migrated into the ganglion cell layer and inner nuclear layer, as shown by white arrowheads (F). The number of Iba1+ immune cells infiltrating into the GCL and INL was quantified after masking the identify of each image (G). Images representative of sections from 3 control and 3 diabetic mice. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Scale bar = 20 µm. ** = $p<0.01$.

The development of oxidative stress in the diabetic retina is a key stage in the pathophysiology of diabetic retinopathy (Kowluru & Chan 2007), where it is associated with activation of pro-inflammatory and pro-angiogenic signalling pathways (Kim et al. 2013; West et al. 2010). 4-Hydroxynonenal (4-HNE), a marker of lipid peroxidation, is elevated in conditions of oxidative stress. Hence making it a good marker for oxidative stress-induced cellular changes.

To determine whether oxidative stress was increased in the diabetic retina, cryosections were stained for 4-HNE and analysed by IF (Figure 20). 4-HNE staining appeared to be increased in the diabetic retina (Figure 20 D-F), compared to the control retina (Figure 20 A-C) with the greatest increase in 4-HNE staining in the photoreceptors. Quantitative image analysis revealed a 3-fold increase in 4-HNE staining in diabetic retinas compared to controls (Figure 20 G). This is consistent with a previous publication which showed that photoreceptors are a major contributor to oxidative stress in the diabetic retina (Du et al. 2013), and provides further evidence of early pathological changes in the diabetic retina.

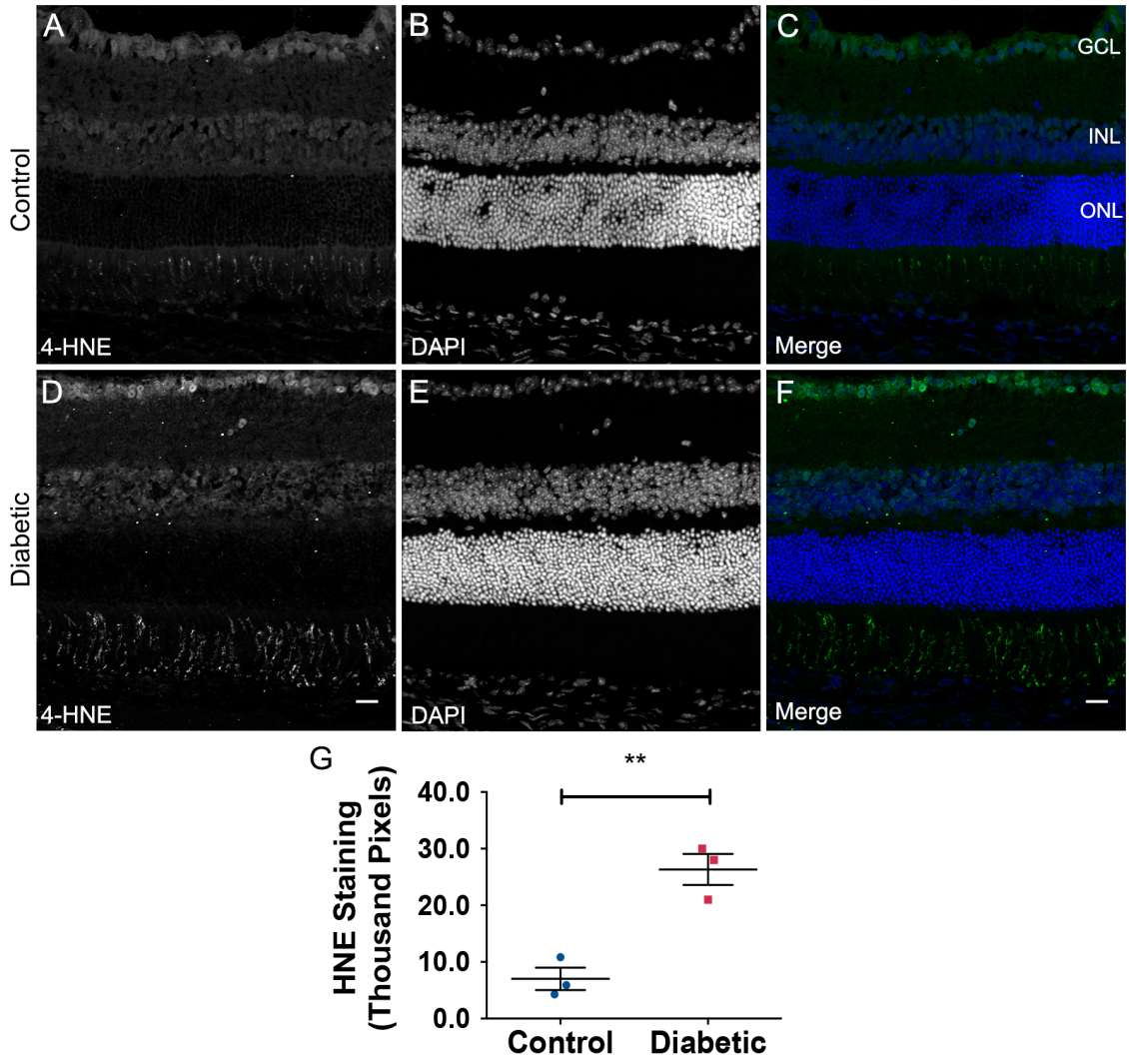

Figure 20: 4-HNE staining in the control and diabetic mouse retina. 12 µm cryosections were stained for 4-HNE to examine oxidative stress-induced changes to the retina in control (A-C) and diabetic (D-F) mice two months after the induction of diabetes. The amount of 4-HNE staining appeared to be increased in the photoreceptors (located below the ONL) in sections from diabetic retinas, also indicated by quantitative image analysis (G), which revealed a statistically significant increase in 4-HNE staining in diabetic retinas. Images representative of sections from 3 control and 3 diabetic mice. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Scale bar = 20 µm. ** = $p<0.01$.

NF-κB is a transcription factor which induces the transcription of various pro-inflammatory cytokines such as *Tnfa*, *Il1beta* and *Il6* in response to pro-inflammatory signalling (Hoesel & Schmid 2013). It is known to be involved in diabetic retinopathy, where it's induced by Vegf and other cytokines (Barlovic et al. 2011; Bai et al. 2009; Tang & Kern 2011). The levels of the phosphorylated p65 subunit of NF-κB were measured by IHC and western blot to examine the inflammatory state of the retina in control and diabetic mice (Figure 18).

Retinal cryosections were stained for the p65 subunit of NF-κB to see if NF-κB signalling was activated by entry of p65 to the nucleus (Figure 21). Both control and diabetic (Figure 21 A-F) retinas were examined for co-localisation between DAPI and NF-κB, however, there was no evidence of co-localisation in either case. In addition to analysis of NF-κB activation by IHC, WB analysis of NF-κB was also performed. Whole retinal tissue lysates were prepared and analysed by western blot for the ratio of phosphorylated NF-κB p65 to total NF-κB p65 with appropriate phospho- specific and pan NF-κB p65 antibodies. In contrast to NF-κB p65 analysis by IHC, the amount of phosphorylated NF-κB p65 was significantly increased ($p<0.05$) in lysates from diabetic retinas compared to controls (Figure 21 G-H). This shows that NF-κB signalling is increased in the diabetic retinas compared to controls. Whilst the results from these two methods of analysis are at odds, it is likely that the antibody preferentially binds epitopes only exposed upon denaturation.

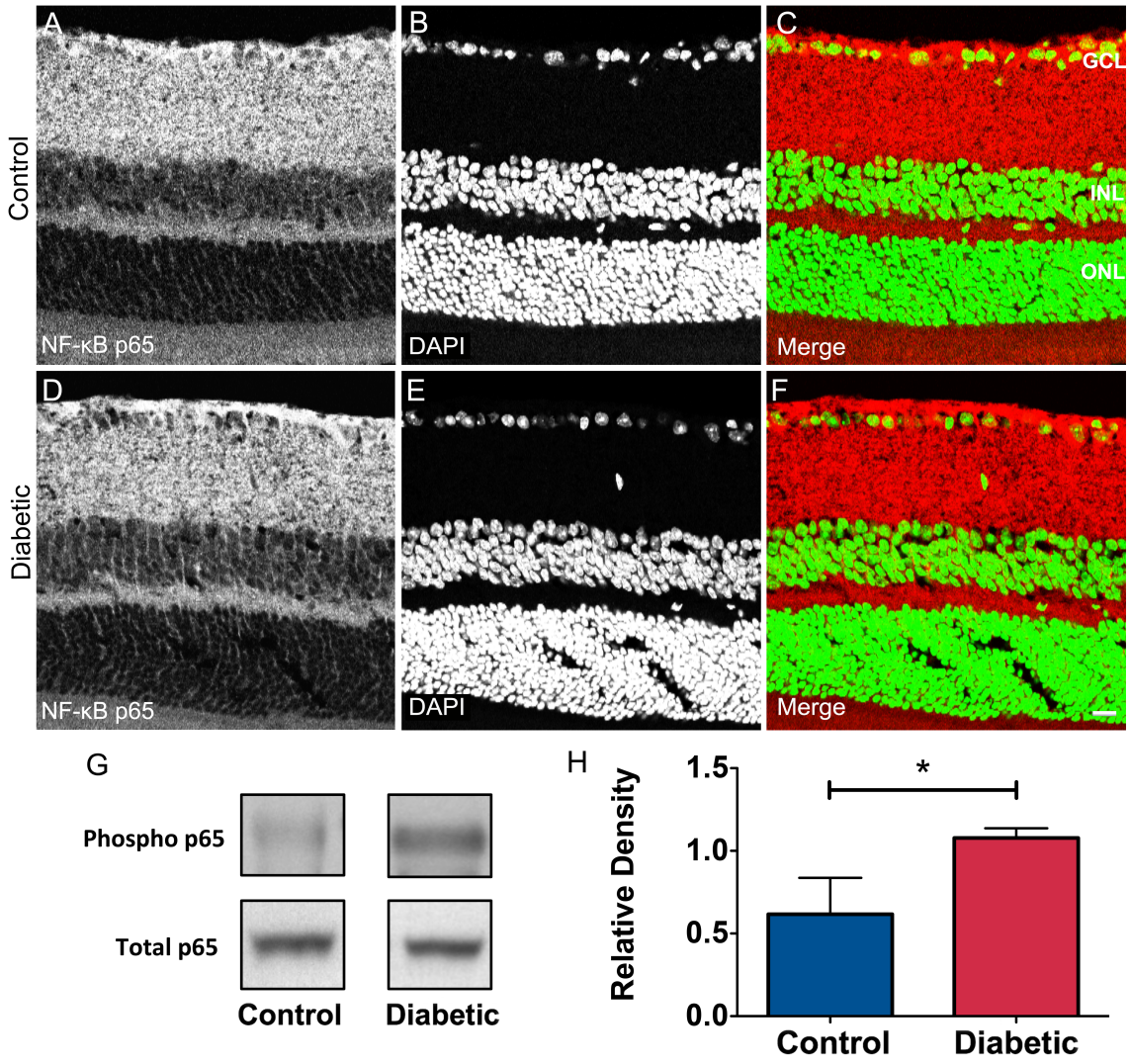

Figure 21: Analysis of NF-κB p65 in the retinas of control and diabetic mice Cryosections from control (A-C) and diabetic (D-F) mice two months after diabetes induction were stained for Nf-κb (A, D) and DAPI (B, E). No meaningful evidence of Nf-κb nuclear translocation was observed in control or diabetic mice. Representative blots probed with antibodies to phosphorylated Nf-κb p65 and total Nf-κb p65 (G). Quantification of band intensities by normalising the intensity of phospho-p65 blots to the intensity of total-p65 blots (H) revealed that the amount of phosphorylated Nf-κb p65 was increased in diabetic retinas compared to controls. Images are representative of sections from 3 control and 3 diabetic mice. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Scale bar = 20 μm. Western blot data from three control and three diabetic mice. * = $p<0.05$. Error bars = S.E.M.

Vascular leakage is a key feature of diabetic retinopathy and is associated with progression of disease severity, being a particularly destructive complication of DR (Roy et al. 2017). Additionally, the onset of vascular leakage and development of macular edema is a clinical indication for the use of anti-VEGF therapeutics such as aflibercept (Cheung et al. 2010). Whilst no vascular leakage was detected by fluorescein angiography (Figure 14), publications investigating vascular leakage have utilised other methods such as perfusion with fluorescently labelled tracers (Wang et al. 2010) and staining for endogenously secreted albumin (Xin et al. 2013).

To examine vascular leakage in the retina in greater detail, cryosections were stained for IgG with a mouse-specific Alexa-Fluor-488 conjugated secondary antibody to label the vasculature and detect areas of vascular leakage. Low power images of control and diabetic (Figure 22 A, D) retinas two months after diabetes induction show that whilst areas of leakage were relatively rare in diabetic retinal sections, they were completely absent in sections from control retinas. High power images of control (Figure 22 B-C) and diabetic (Figure 22 E-F) samples reveal that whilst there was some positive staining for IgG in control retinas, it was limited, as expected, to the retinal vasculature. In contrast, in diabetic retinas there was a greater amount of IgG staining which appeared to result from extra-vascular IgG, and is therefore evidence of vascular leakage. Quantitative image analysis of IgG staining (Figure 22 G) revealed a 20-fold increase in IgG staining in diabetic retinas, however, due to the variability in IgG staining between samples this observation was not statistically significant. Further analysis

of more samples would be required to improve the statistics in this experiment.

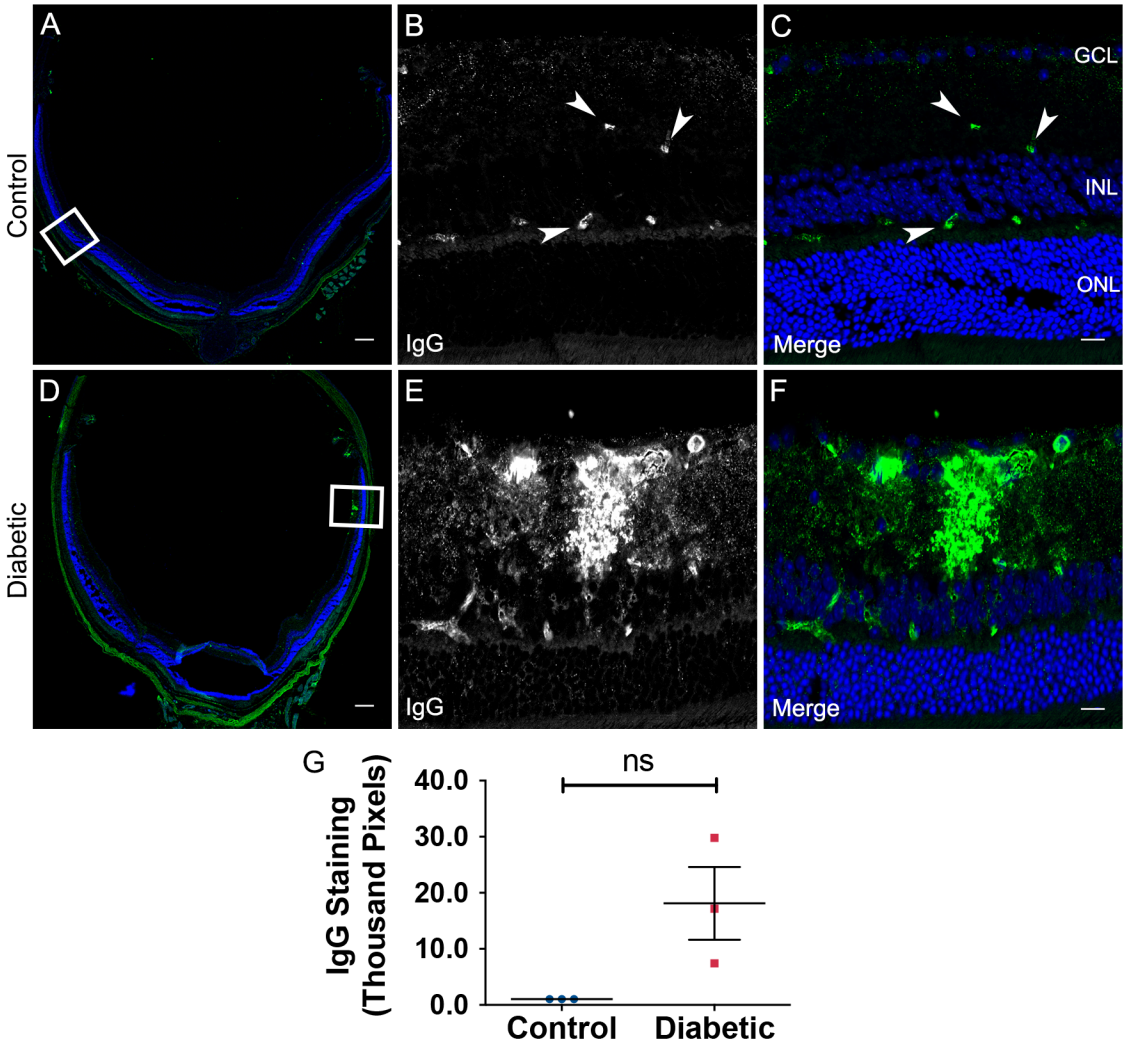

Figure 22: Evidence of vascular leakage of IgG in diabetic mouse retinas. Two months after the induction of diabetes, retinas were isolated and 12 µm cryosections were stained for mouse IgG. Low power images of control (A) and diabetic (D) mouse retinas. White boxes shows areas of interest for high power images in (B-C) and (E-F). IgG staining was increased in diabetic (D-F) retinas compared to controls (A-C), where IgG staining was limited to the retinal vasculature (arrowheads). Quantitative image analysis of IgG staining in control and diabetic retinas (G). Images representative of sections from 3 control and 3 diabetic mice. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Scale bar = 20 µm.

Analysis of altered GFAP expression in retinal cryosections did not reveal any striking differences between control and diabetic animals (Figure 18). Therefore, GFAP staining in mouse retinal flatmounts was analysed in order to investigate potential changes to astrocytes and their interaction with the vasculature in the diabetic retina. Flatmounted retinas were stained for both GFAP and collagen IV (Figure 23 A-F) and quantitative image analysis of the overlap between collagen IV and GFAP staining was performed to determine the extent of astrocyte interaction with the vasculature (Figure 23 G), however, there was no significant difference in the amount of GFAP overlap with collagen IV. Next, to analyse any changes to the distribution of astrocytes in the retina, binary images were generated from GFAP IF images and converted to show the outlines of the astrocytes (Figure 23 H-I). The identity of the images was masked and the number of astrocytes in each image was manually counted (Figure 23 J). However, no significant difference in astrocyte number was found.

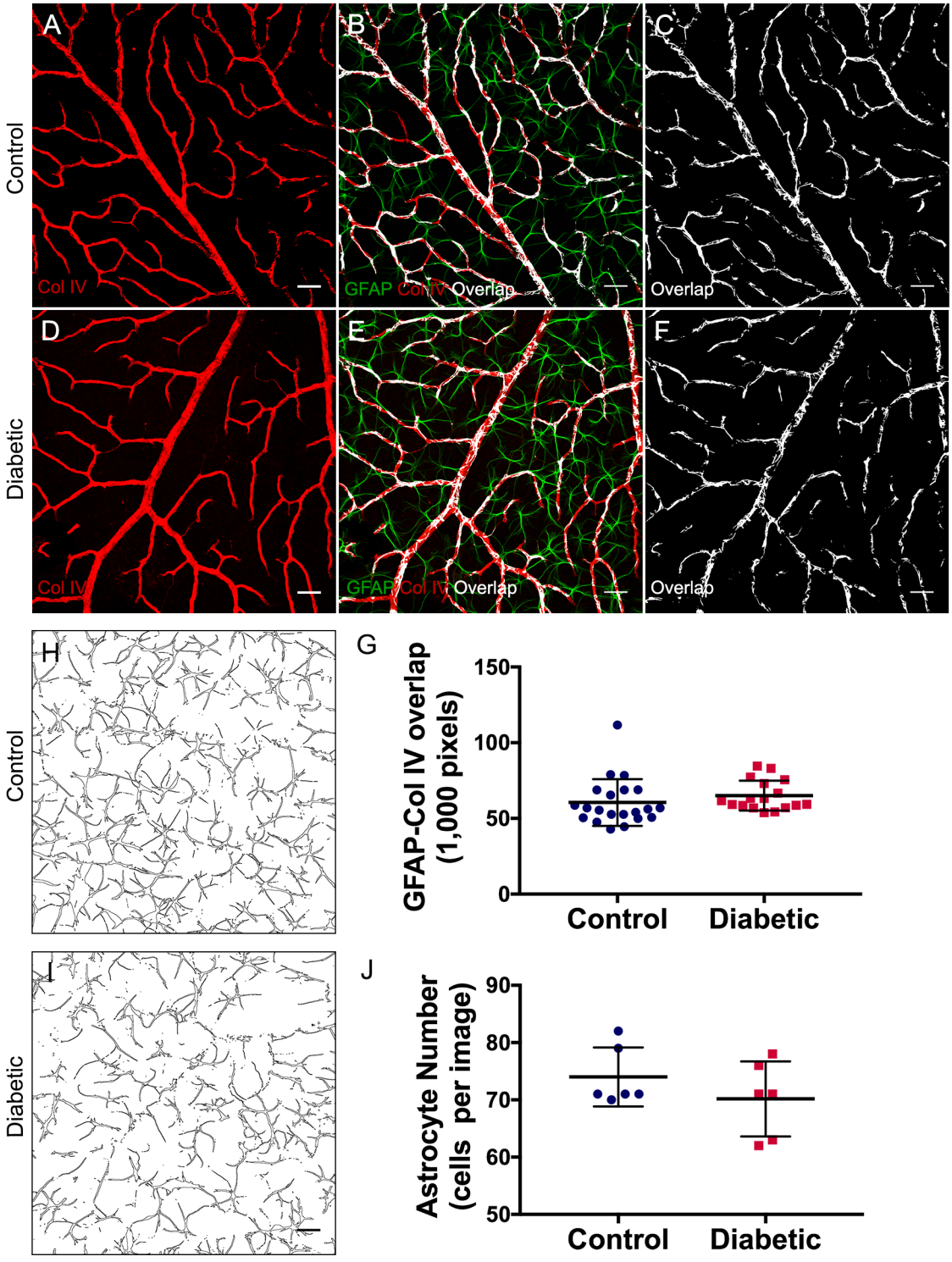

Figure 23: Astrocyte-endothelial cell interactions in the diabetic retina. Flatmounted retinas were isolated two months after the induction of diabetes from control (A-C) and diabetic (D-F) mice and stained for collagen IV and GFAP. Quantitative image analysis of the overlap of collagen IV and GFAP staining (G) revealed no significant difference in the degree of overlap of these two markers. Outlines of GFAP positive cells were generated from

binary images of GFAP staining (H-I). Cells were counted manually after the being blinded, which revealed that there was no significant difference in astrocyte number in control or diabetic mice (J). For analysis of collagen IV and GFAP overlap, 3 different images from 6 mice from each group were used for image analysis. For astrocyte number, one image from 6 mice from each group were used. Scale bar = 50 μm.

Having identified an increase in extravascular IgG staining in the sections of diabetic retinas (Figure 22), I wanted to determine what the pattern of vascular leakage looked like across the whole retina. Since vascular leakage is not uniform across the diabetic retina, analysing tissue sections cannot give a complete picture of the full extent of vascular leakage. IgG has a relatively high molecular weight for a circulating protein, so I chose to examine the leakage of 40 kDa FITC-dextran as I reasoned this would be a more sensitive indicator of vascular leakage than endogenous IgG staining. FITC-dextran was intravenously administered to mice via the tail vein and allowed to circulate for 1 hour before mice were euthanised and retinas were processed for analysis by IF. However, imaging of retinas from control and diabetic mice revealed no evidence of vascular leakage after perfusion with FITC-dextran (Figure 24 A-B). In contrast, when endogenous IgG staining was analysed in retinal flatmounts by IF, this revealed increased extravascular staining for IgG in diabetic retinas (white arrowheads, Figure 24 C-D), consistent with the data obtained from retinal sections (Figure 22).

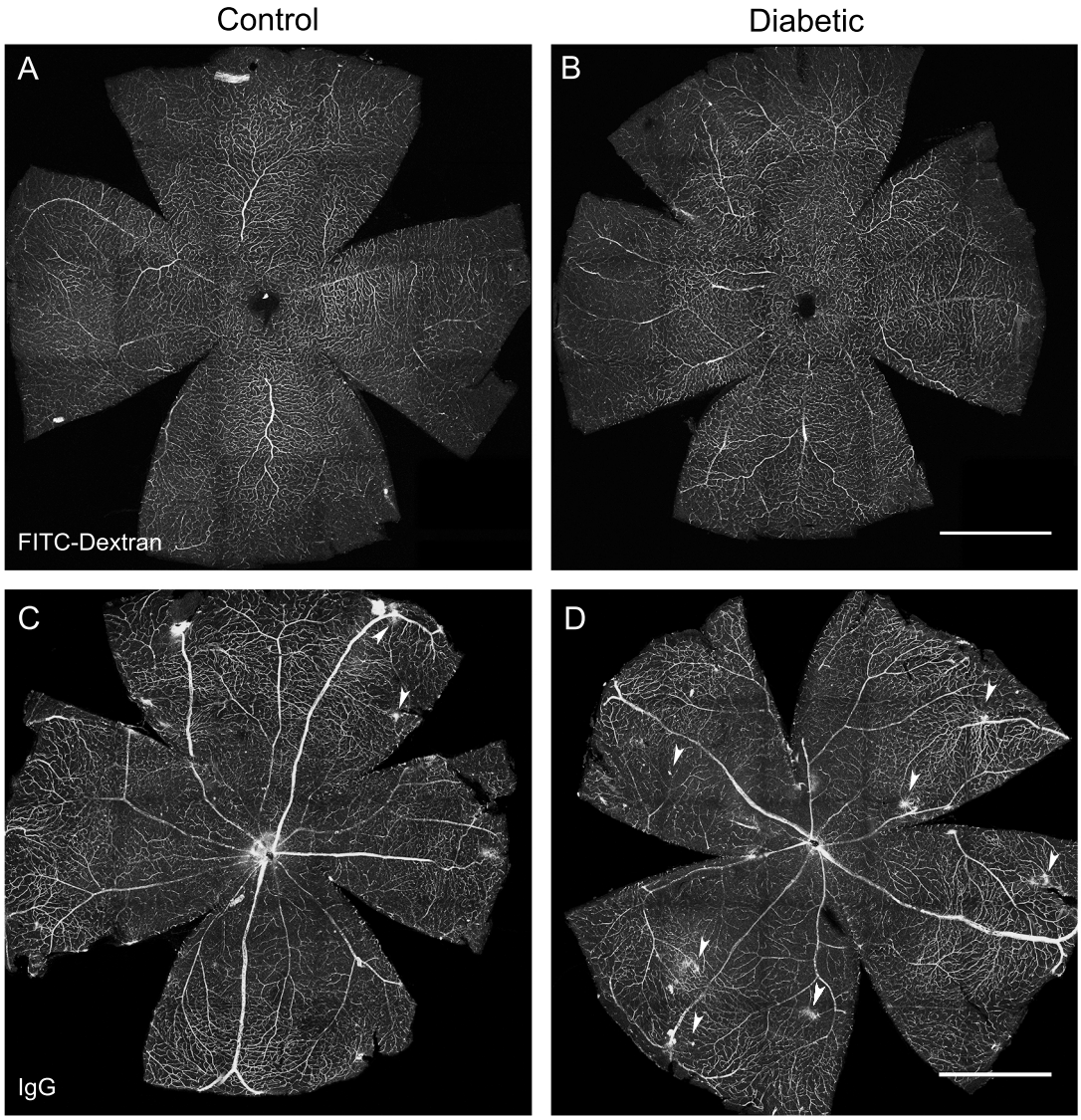

Figure 24: Assessment of vascular leakage in retinal flatmounts. Staining for endogenous IgG revealed areas of vascular leakage in retinal flatmounts, that were not observed following intravenous perfusion of FITC-dextran. Two months after the induction of diabetes, control or diabetic mice were perfused with 100 µg/mL of 40-kDa FITC-dextran for 1 hour, before being euthanised (A-B) to examine vascular leakage. Alternatively, flatmounted retinas were stained for endogenous IgG by IF (C-D). There was no evidence of extravascular FITC-dextran in diabetic retinas (B), however, there was an increased amount of extravascular IgG in diabetic retinas (white arrowheads, D) compared to control retinas (C). Scale bar = 1 mm. Images representative of 6 mice per group.

2 Gene expression in the streptozotocin-induced diabetic mouse retina

Altered expression of pro-inflammatory and pro-angiogenic genes in diabetic retinopathy plays a key role in changes to the retinal microenvironment in diabetes. To examine whether gene expression was altered in the retinas of diabetic mice, RNA was extracted from retinas from diabetic mice and appropriate age-matched controls two months after diabetes induction and gene expression was analysed by qPCR. The role of some growth factors in diabetic retinopathy, such as Vegf, has been well characterised, whilst the roles of other genes that were examined have yet to be determined. Comparing the expression of Vegf with other genes such as angiopoietin like 2 (Angptl2), angiopoietin like 6 (Angptl6) and Lrg1 will help determine the roles they play in diabetic retinopathy and whether they might be considered as potential new therapeutic targets.

As has been previously described (Bai et al. 2009), *Vegf* expression was increased in diabetic retinas compared to controls (Figure 25 A). This highlights the important role of Vegf and vascular leakage in diabetic retinopathy, and is consistent with data in Figure 22 & Figure 24. Whilst LRG1 is increased in the vitreous of diabetic patients, and has been demonstrated to promote angiogenesis and vascular leakage *in vitro* and *in vivo* (Wang et al. 2013), its role in animal models of diabetes has not yet been examined. Both *Lrg1* and *Tgfbeta* were increased in the diabetic retinas (Figure 25 E-F).

The role of the Ang-1 and Ang-2 in regulating angiogenesis and vascular permeability is well established. However, the family of angiopoietin-like proteins (Angptl) has 9 members which signal independently of Tie1/Tie2 receptors with various effects on the vascular endothelium (Hato et al. 2008) and are generally less well understood. The roles of various proteins of this family have recently been discovered in the retina. Angptl-4 was shown to promote retinal vascular permeability, and was upregulated in hypoxic conditions (Xin et al. 2013). The roles of Angptl-2 and Angptl-6 in the diabetic retina have not yet been characterised, so I decided that these were two genes worth investigating with a potential role in diabetic retinopathy.

Angptl2 has previously been shown to mediate inflammation in the endotoxin-induced uveitis model of retinal inflammation (Kanda et al. 2012). Similarly to *Vegf*, *Angptl2* expression was increased in diabetic retinas compared to controls (Figure 25 B). Angptl6 has been previously shown to induce angiogenesis and vascular permeability *in vivo* (Oike et al. 2004). Whilst *Angptl6* is predominantly expressed by the liver, an alternative *Angptl6* transcript encoding exons 4-6 is more widely expressed in a number of different tissues (Hato et al. 2008). In the mouse retina, the relative expression of the transcript encoding exons 4-6 was approximately 100-fold higher than the expression of the full length *Angptl6* transcript (Figure 25 C-D). Unlike the full-length transcript (Figure 25 C), the transcript encoding exons 4-6 of *Angptl6* was expressed at a statistically significantly higher level in diabetic retinas compared to controls (Figure 25 D).

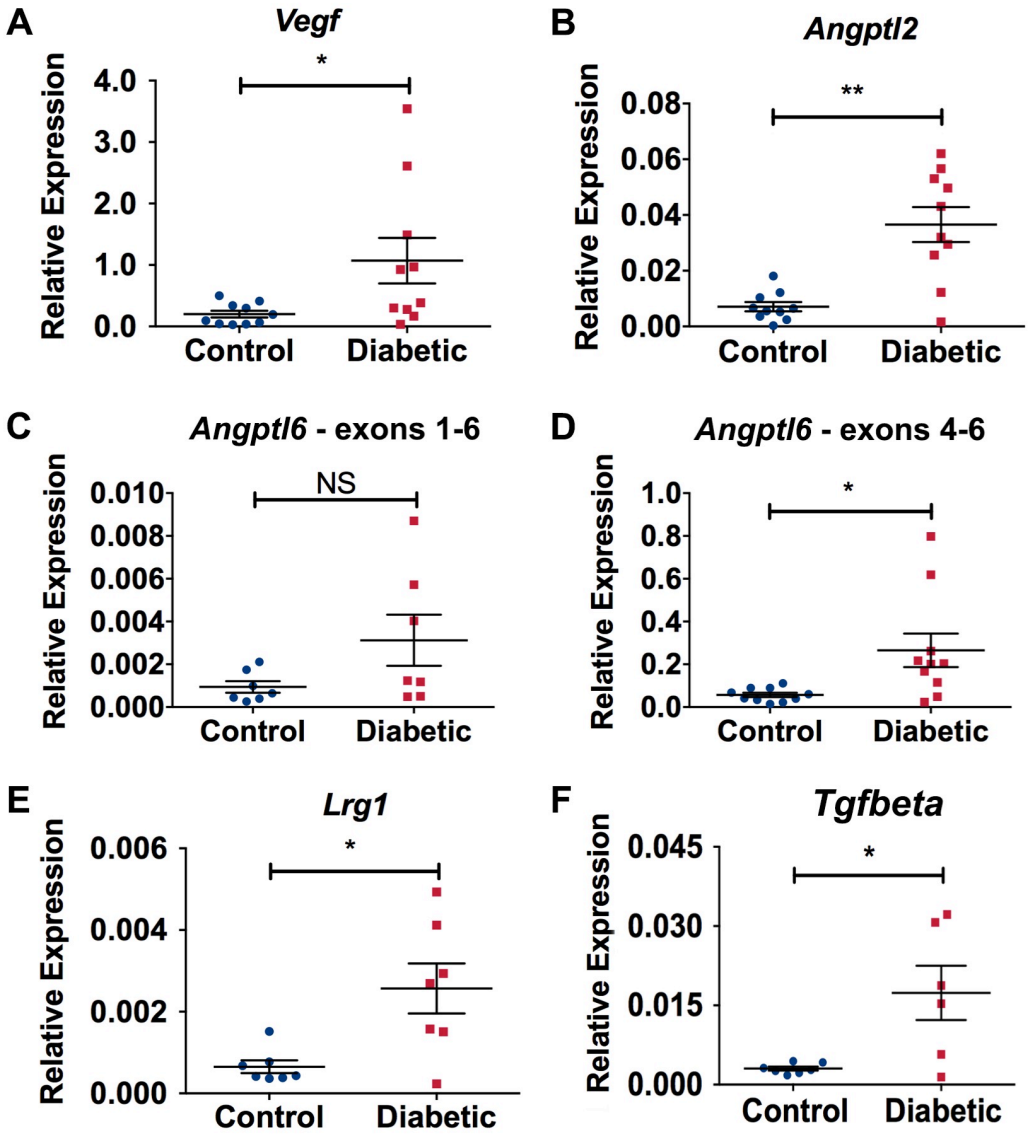

Figure 25: Altered gene expression in control and diabetic mouse retinas. RNA was extracted from control or diabetic mouse retinas for qPCR analysis two months after diabetes induction. qPCR showed a statistically significant increase in the levels of *Vegf* (A), *Angptl2* (B), *Lrg1* (E) and *Tgfbeta* (F) in diabetic retinas. Primers that targeted the full-length transcript for *Angptl6* (C) showed there was no increase in expression of the full-length transcript, whilst primers that targeted the transcript of exons 4-6 (D) showed a statistically significant increase in expression in diabetic mice. Additionally, expression of the shorter transcript was higher than that of the full-length transcript in both the control and diabetic retinas (C, D). Data from at least 6 experimental animals per group. Statistical analysis was performed using students T-test with Welch's correction to control for unequal variance. * = $p<0.05$. ** = $p<0.01$.

3 Angptl6

The differential expression of the two Angptl6 transcripts indicates that the regulation of this gene differs according to the tissue and cell type. The functional domains of Angptl6 appear to be present in the N-terminus of this protein, which indicates that both the full length and truncated isoform have similar biological activity. As *Angptl6* expression has not previously been reported in the retinas of diabetic mice, I wanted to test whether its pro-angiogenic effect would be evident in the mouse metatarsal assay (Figure 26). This would enable me to further characterise the effect of Angptl6 on the vasculature.

However, quantification of angiogenesis by analysis of CD31 staining revealed that treatment with 1 µg/mL of recombinant human ANGPTL6 did not reveal any increase in angiogenesis (Figure 26), despite this concentration being previously reported in the literature to be sufficient to induce angiogenesis (Okazaki et al. 2012; Oike et al. 2004). In contrast, VEGF which was used here as a positive control (Figure 26 C) showed a pro-angiogenic response. It was disappointing to find that in our hands ANGPTL6 did not induce angiogenesis as had previously been reported *in vitro* and *in vivo* (Oike et al. 2004), although this may have been due to the assay utilised, or the human protein may be less bioactive on mouse cells.

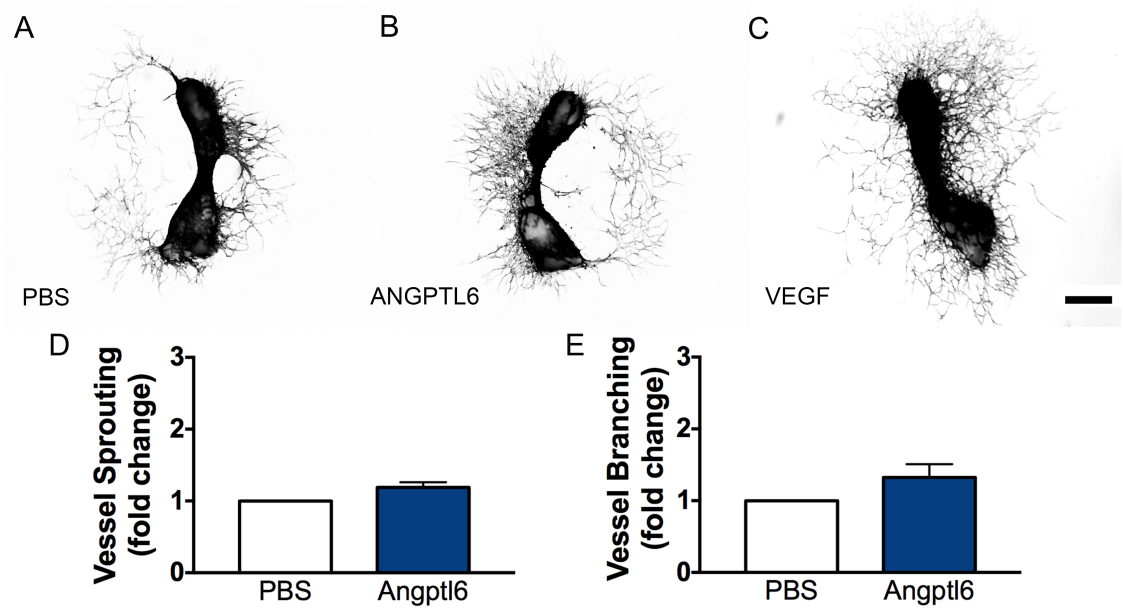

Figure 26: Recombinant human ANGPTL6 does not promote angiogenesis in the mouse metatarsal assay. Metatarsals were cultured for 10 days in basal media or with 1 µg/mL recombinant ANGPTL6. Cultured metatarsals were stained for CD31 to label endothelial cells (A-C). CD31 staining was analysed to measure the amount of vessel sprouting and branching (D-E). Untreated (A) or ANGPTL6 at 1 µg/mL (B) showed no change in vessel formation (D) or vessel branching (E). There was increased angiogenesis when metatarsals were treated with 50 ng/mL of VEGF as a positive control (C). Data from 2 independent experiments with at least 12 bones per experiment. Scale bar = 500 µm.

4 Lrg1

Of the various factors investigated, neither Angptl2 nor Angptl6 were considered to be sufficiently promising for further study. However, the increase in *Lrg1* expression in the retinas of diabetic mice (Figure 25 E), together with previously published observations that LRG1 is increased in the vitreous of patients with PDR (Wang et al. 2013) suggests a possible role for Lrg1 in diabetic retinopathy. To further explore the role of Lrg1 in the streptozotocin mouse, diabetes was induced in Lrg1 KO mice and age-matched WT controls to determine whether the absence of Lrg1 would alter the phenotype of the diabetic retina.

In the first instance, fundus images were taken to examine whether there were any changes to the diabetic retina in Lrg1 KO mice versus WT controls two months after diabetes induction. Compared to WT animals (Figure 27 A-B), the abnormal retinal phenotype appeared to be less severe in the Lrg1 KO mice with fewer hyper-reflective spots (Figure 27 C-D). This suggests that Lrg1 KO mice may have a less severe diabetes-induced retinal phenotype. It is important to note that in this cohort, the WT diabetic mice (Figure 27 A-B) had a more severe retinal phenotype than their equivalent WT diabetic counterparts in Figure 13. It should also be noted that the Lrg1 KO mice and their WT littermate controls (mice were bred from heterozygous pairs) were bred and housed in what was designated as a 'clean room' in the animal facility, and hence may be more sensitive to diabetes-induced inflammatory activation of the immune system.

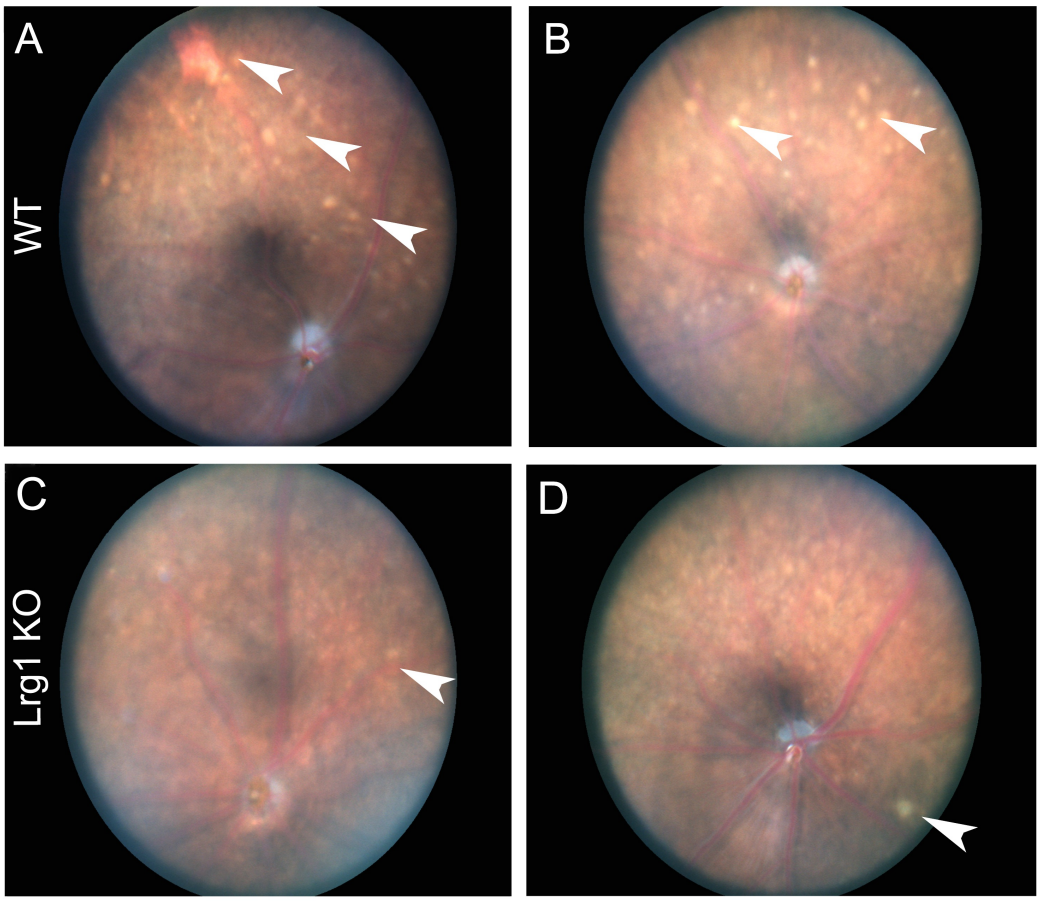

Figure 27: Fundus imaging in WT and Lrg1 KO diabetic mice. Two months after diabetes induction, fundus imaging of diabetic retinas from WT (A-B) and Lrg1 KO (C-D) mice revealed that the KO mice had a less severe diabetic phenotype (white arrowheads) in the retina, as judged by a reduction in the number of inflammatory infiltrates.

Having shown that Lrg1 KO mice have a less severe retinal phenotype after the induction of diabetes, the levels of blood glucose were compared with WT diabetics to ensure the severity of diabetes was similar across different strains (Figure 28). The similar blood glucose levels show that any differences in retinal phenotype are therefore likely to be due to the different genotypes rather than differences in the severity of hyperglycaemia.

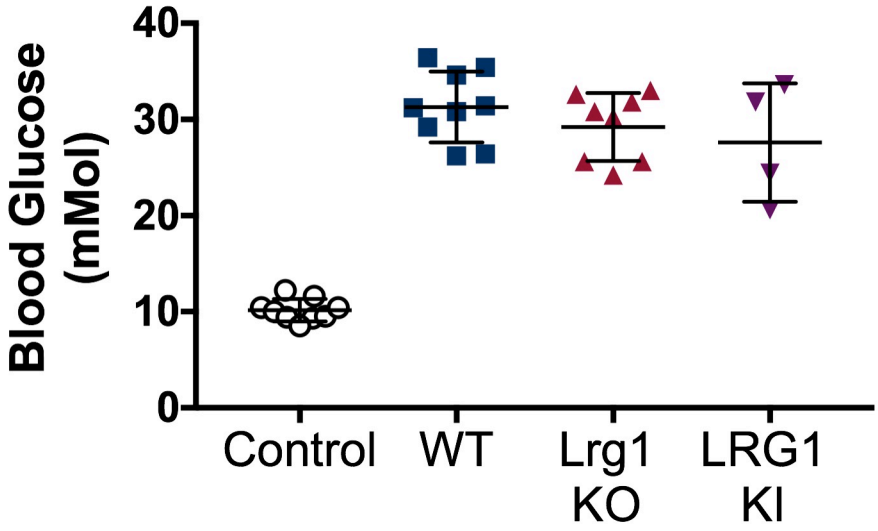

Figure 28: Blood glucose measurements in diabetic mice. Blood glucose was measured using a glucometer two months after diabetes induction. Blood glucose levels were similar in WT, Lrg1 KO and human LRG1 knock-in (KI) mice.

Having shown that staining retinal flatmounts for endogenous IgG is a more reliable way of examining vascular leakage than perfusing with fluorescent dextrans (Figure 24) or by fluorescein angiography (Figure 17), and that diabetic Lrg1 KO mice apparently have a milder retinal phenotype (Figure 27), I decided to investigate whether vascular leakage was reduced in diabetic Lrg1 KO mice. Retinas from WT or Lrg1 KO mice were stained for IgG, two months after the induction of diabetes to examine vascular leakage. Vascular leakage appeared to be reduced in the retinas from diabetic Lrg1 KO mice, compared to diabetic WT mice, comparison of the number of leakage foci revealed a statistically significant ($p<0.05$) decrease in the number of leakage sites in Lrg1 KO mice versus WT controls (Figure 29). This indicates that the absence of Lrg1 attenuates diabetes-induced vascular leakage in the retina, in agreement with previous data which has

demonstrated that it plays a role in increased vascular permeability and angiogenesis in other disease models (Wang et al. 2013).

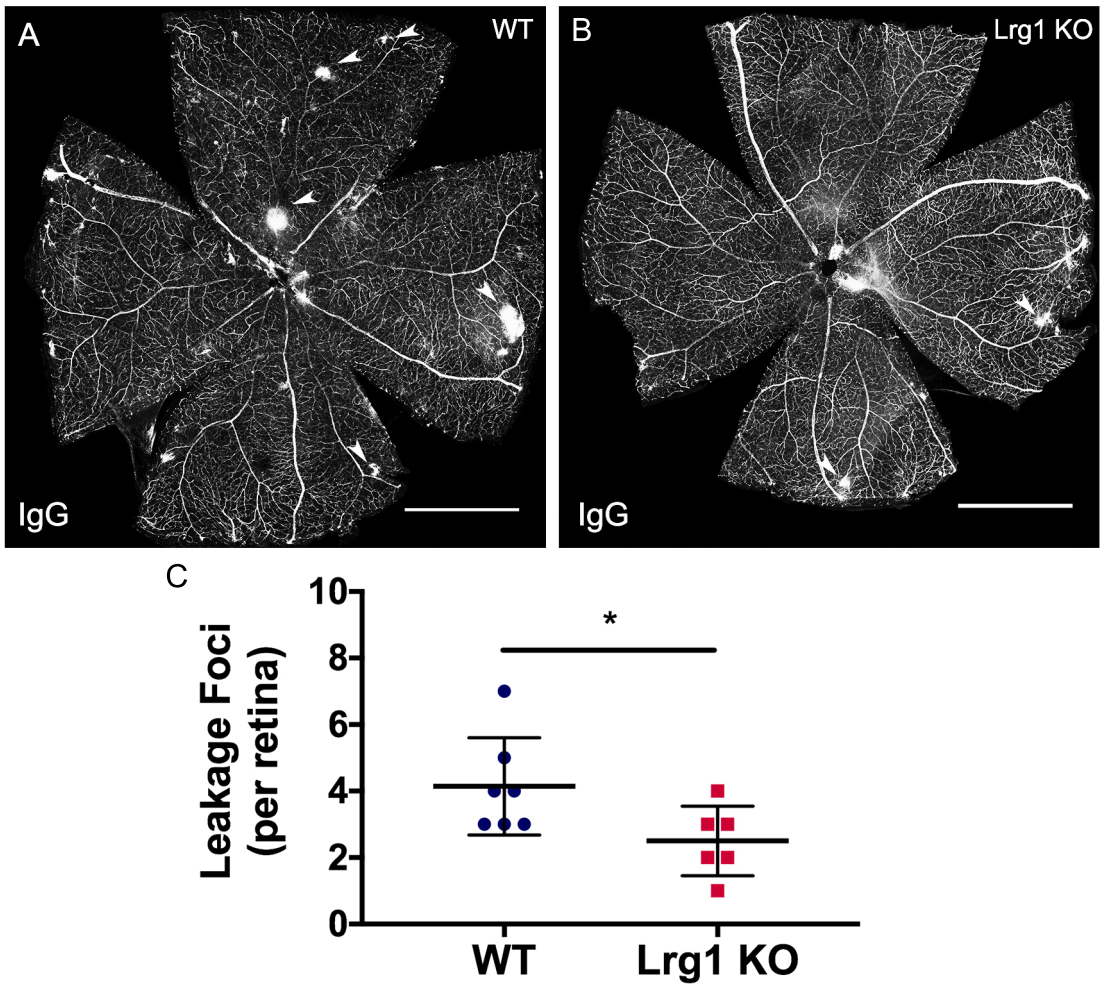

Figure 29: Vascular leakage in diabetic WT and Lrg1 KO mice. Flatmounted retinas from WT (A) or Lrg1 KO (B) mice two months after the induction of diabetes were stained for endogenous IgG. The number of leakage foci were counted manually after being blinded to the identify of the images (C), which revealed a statistically significant decrease in the sites of vascular leakage in Lrg1 KO mice. Representative images from 6-7 mice per genotype. Scale bar = 1 mm. Statistical analysis was performed using students T-test. * = $p<0.05$.

Having shown that diabetes-induced retinal vascular leakage is attenuated in the Lrg1 KO mice, I sought to examine the effect of diabetes on the human LRG1 knock-in (KI) mouse. This KI mouse was generated in order to test the efficacy of a humanised LRG1 blocking antibody as a potential therapeutic, as the antibody does not cross-react with the mouse protein. Additionally the human *LRG1* transgene is flanked by *LoxP* sequences, which allows it to be deleted by the CRE recombinase, permitting cell/tissue specific deletion of the gene. The ability to conditionally knock out the gene in the mouse would therefore allow further mechanistic insight of the role of Lrg1 in the retina.

Since human LRG1 KI mice have an increased blood glucose level after diabetes induction, similar to Lrg1 KO and WT mice (Figure 28), one would expect these mice to respond in a similar manner as WT mice to the induction of diabetes. Vascular leakage was analysed two months after the induction of diabetes in hLRG1 KI mice (Figure 30). IgG staining revealed that diabetic hLRG1 KI mice had increased vascular leakage compared to age-matched non-diabetic controls, as has been previously demonstrated in WT mice. This shows that hLRG1 KI mice develop a similar retinal phenotype in response to streptozotocin-induced diabetes as occurs in WT mice. Another advantage of this mouse strain is its compatibility with the human LRG1 ELISA to detect changes in the level of LRG1 protein in the retina, as there is a lack of suitable reagents to develop a mouse Lrg1 ELISA. Having previously demonstrated by qPCR that *Lrg1* is upregulated in the mouse retina (Figure 25 E), I sought to demonstrate that LRG1 protein is also upregulated in the diabetic LRG1 KI retina. Although an ELISA was

performed to analyse LRG1 in control and diabetic mouse retinas, the concentration of LRG1 in whole retinal lysates was below the lower limit of detection of this assay.

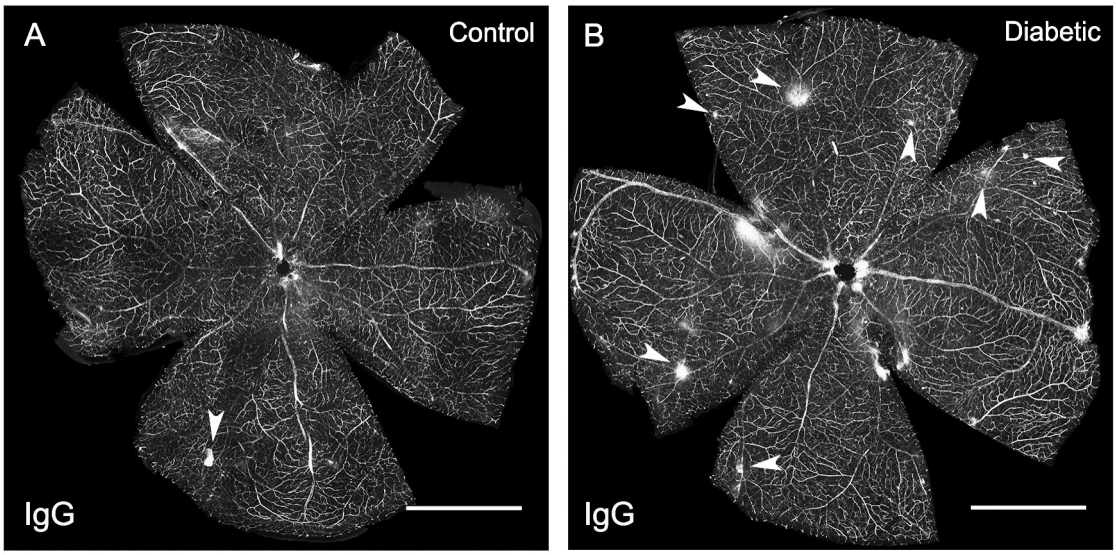

Figure 30: Human LRG1 KI mice develop symptoms of diabetic retinopathy similarly to WT mice. Vascular leakage was analysed by endogenous IgG staining two months after diabetes induction in control (A) or diabetic (B) LRG1 KI mice. Images representative of 4 mice per condition. Scale bar = 1 mm.

In summary, work in this chapter has shown that although the streptozotocin mouse fails to develop the severe retinal phenotype observed in other models and strains, it does nevertheless exhibit a wide range of sub-pathological changes. As a tool for investigating early changes this model therefore offers several advantages, since a primary goal of therapeutic intervention in human disease is the prophylactic control of PDR and DME in patients already at risk.

Chapter 5

Discussion

1 Effect of hyperglycaemia on angiogenesis *in vitro*

In the first instance, the aim of the work in this thesis was to investigate the effects of hyperglycaemia on the vasculature *in vitro*, using the metatarsal assay. One of the major limitations of *in vitro* angiogenesis assays is that it is difficult to recreate the complex intercellular interactions between different cell types and the structural ECM components that exist *in vivo*. To address these limiting factors, the embryonic mouse metatarsal assay is an effective *ex vivo* model of the vasculature, which acts as a compromise between a simpler *in vitro* study with cultured cells and a more complex *in vivo* one (Song et al. 2015).

These studies in the metatarsal assay involving the supplementation of culture media with additional levels of glucose showed that high glucose treatment (25 mM) led to a reduction in angiogenesis. Prior to the development of neovascularisation in diabetic retinopathy, the behaviour of the vasculature and the endothelial cells is altered. Whether these changes are due to the direct effect of elevated blood glucose or glycated protein adducts on the endothelial cells themselves, or elicited by paracrine mediators from surrounding cells has not been firmly established, although it is feasible that all play a role.

In this assay, high glucose did not induce changes in the ratio of pericyte-endothelial cell staining, which is known to be reduced in the diabetic retina (Pfister et al. 2008). This implies that in this assay, high glucose may primarily alter the behaviour of endothelial cells rather than the perivascular cells. However, effects on other perivascular cells, such as smooth muscle

cells, macrophages and fibroblasts, may also occur in response to high glucose but these were not examined in this study. Whilst the relationship between endothelial cells and pericytes has been well characterised, especially in the context of diabetic retinopathy, less is known about the role of the other perivascular cells and their association with diabetes induced changes to the retinal vasculature. Further work to investigate these changes could identify key cellular changes in diabetes and expand the knowledge in this area of research. Since many of these cells are present in the metatarsal assay, this would be an ideal model system to investigate this, as high glucose and other diabetic insults could be used to induce these changes a the cellular level.

One explanation for the reduction in angiogenesis may be due to high glucose induced upregulation of the Notch ligand, Jagged 1 (Jag-1), as has been previously described in a co-culture model of endothelial cells and smooth muscle cells (Yoon et al. 2014). Jag-1 is pro-angiogenic during development, and antagonises the notch ligand Delta-like 4 (Dll4), which attenuates Dll4/Notch mediated vessel stabilisation and maturation (Benedito et al. 2009). The high glucose-induced increase in Jag-1 was accompanied by an initial increase in sprouting and branching of unstable vessel sprouts which later regressed, unlike cultures treated with mannitol which sprouted normally (Yoon et al. 2014). Jag-1 has also been demonstrated to play a role in diabetes-induced capillary degeneration in the retina and myocardium, EC specific knockdown of Jag-1 led to a reduction in diabetes-induced capillary degeneration (Yoon et al. 2016). However, another group has shown that Jag-1 is decreased in smooth-

muscle cells in hyperglycaemia which is anti-angiogenic (Lee et al. 2016). Therefore one could expect Jag-1 to behave differently according to the cell type and context. Whilst Jag-1 is pro-angiogenic during development, its aberrant activation in the mature vasculature in pathological conditions leads to the disruption of the vasculature.

High glucose caused a statistically significant reduction in Vegf secretion in the metatarsal assay, in agreement with other reports where Vegf was reduced in endothelial cells exposed to high glucose (Yang et al. 2008; Yu et al. 2016). Compared to these other reports, the reduction in Vegf in response to high glucose was relatively modest, with approximately 25% reduction in Vegf secretion observed in this data set compared to a 75% decrease as reported by (Yang et al. 2008; Yu et al. 2016). Whether or not a 25% reduction in Vegf production has a physiological function remains unclear, to determine this, one could titrate in exogenous Vegf to see if it alters the level of angiogenesis in the metatarsal assay. However, high glucose treatment has been shown to induce Vegf in other cell types such as fibroblasts, smooth muscle cells and pericytes (Doronzo et al. 2012; Tsai et al. 2013; Giurdanella et al. 2015). Therefore, it is plausible that high glucose could be decreasing Vegf expression in the endothelial cells in this assay whilst increasing Vegf expression in other cell types. This could explain why a modest decrease in Vegf secretion was observed. To determine the effect of high glucose on Vegf expression in different cell types, fluorescent *in situ* hybridisation could be used to identify which cell types express Vegf.

One of the consequences of reduced Vegf production specifically in endothelial cells is an increase in apoptosis (Yang et al. 2008). Endothelial cell specific deletion of VEGF leads to a decrease in survival, which cannot be compensated for by paracrine derived Vegf (Lee et al. 2007). Additionally, treating endothelial cells with a VEGFR2 inhibitor led to a decrease in cell survival which was not observed upon treatment with a VEGF blocking antibody (Lee et al. 2007). This highlights the important role for autocrine Vegf signalling in endothelial cell survival and for maintaining the vasculature. Additionally this implies that VEGFR2 signalling activates different signalling pathways depending on whether the receptor is activated at the plasma membrane or intracellularly. To determine whether high glucose treatment alters autocrine Vegf signalling, one could look at VEGFR2 activation by western blotting or immunofluorescence.

It is possible that the observations in this study may have more relevance to other diabetic vascular complications such as peripheral vascular disease and impaired wound healing, which is characterized by insufficient angiogenesis and a reduction in VEGF (Wirostko et al. 2008). Further studies on the potential role of high glucose-induced alterations to angiogenesis in the metatarsal assay with this in mind will allow further exploration of this idea.

2 Effect of AGE-BSA on angiogenesis in the metatarsal assay

Whilst elevated blood glucose is the underlying cause of vascular complications arising in diabetic patients, supplementing cell culture media with additional glucose does not fully recreate the diabetes-induced changes the vasculature is exposed to *in vivo*. Formation of advanced glycation end-products (AGEs) is an important post-translational modification that occurs in diabetic patients (Barlovic et al. 2011). AGEs form *in vivo* through three main mechanisms; the Maillard reaction, the polyol pathway and lipid peroxidation (Ott et al. 2014). AGE deposition occurs on circulating proteins, the plasma membrane and intracellularly, meaning that diabetes-induced increase in AGE formation modifies extracellular and intracellular proteins and other macromolecules.

There are multiple receptors for AGE, which play different functions. Some receptors such as macrophage scavenger receptor and CD36, are involved in the uptake of extracellular AGEs (Araki et al. 1995; Ohgami et al. 2002) which internalises AGEs for detoxification in the lysosome (Grimm et al. 2012). The increase in extracellular AGEs may therefore lead to the accumulation of pathologically high levels of intracellular AGEs, which cannot be degraded. Another important receptor which responds to AGEs is the receptor for advanced glycation end products (RAGE), which plays a key role in activating AGE-induced inflammatory signalling via JAK/STAT, ROS, NF-κB and MAPKs (Ott et al. 2014). Of the many different receptors for AGE, RAGE is the best understood (Ott et al. 2014), and is the only AGE receptor which has been linked to different vascular complications of

diabetes (Li et al. 2011). Based on the current understanding of AGE signalling and the RAGE mediated pro-inflammatory signalling pathways AGE/RAGE signalling is likely to be the primary mechanism for AGE induced pathology in the vasculature and surrounding tissue in diabetes.

Measuring AGE deposition on haemoglobin has superseded blood glucose measurements in the clinic as a biomarker for diabetes severity (Sabanayagam et al. 2009), and whilst AGE deposition on haemoglobin is unlikely to make a significant contribution to AGE/RAGE signalling in endothelial cells and other cell types, it is a useful indicator for the severity of diabetes. Another circulating protein which is modified by AGE deposition is albumin, which has also been linked in the clinic to the severity of diabetes (Vetter 2015). The clinical association of glycated albumin with diabetes and its abundant and free circulation in plasma means that AGE-BSA is a physiologically relevant reagent for studying the effects of AGE. Additionally glycated albumin is a widely used reagent to study the effect of AGEs on the vasculature (Yamagishi et al. 1997; Popov & Simionescu 2006; Wu et al. 2014; Zhang et al. 2015). This means AGE-BSA is a suitable reagent for studying the effects of increased AGE formation on angiogenesis in the metatarsal assay.

Treatment with AGE-BSA led to statistically significant increases in angiogenesis, Vegf secretion and the expression of other pro-angiogenic growth factors, including Il-6. This implies that AGE-BSA induces both angiogenesis and inflammation, the combination of which has been linked to a variety of pathological conditions, including diabetes (Costa et al. 2007;

Kim et al. 2013; West et al. 2010). Also the results from these experiments fit with previous reports which have shown that AGE-BSA is pro-angiogenic and induces VEGF expression *in vitro* and *in vivo* (Yamagishi et al. 1997; Mamputu & Renier 2004; Cohen et al. 2005). Whilst RAGE activation is the most likely cause of increased angiogenesis and growth factor induction in these experiments (Ott et al. 2014), these studies have not resolved whether AGEs from exogenously added AGE-BSA are the sole driver of the altered phenotype in the metatarsal assay, or whether macromolecules on the plasma membrane have also been modified by AGEs or whether AGEs have been internalised by the endothelial cells or other perivascular cells. There is evidence from some *in vivo* studies that AGE-modified albumin is internalised into cells at a higher rate than native albumin in the brain vasculature (Vorbrodt & Dobrogowska 1999), it is therefore plausible that intracellular accumulation of AGE-BSA will occur at a greater rate than native albumin, and that this may have significant effects on the intracellular environment, in addition to the well characterised consequences of extracellularly activating RAGE.

Both previous reports and data in this thesis show that AGEs promote angiogenesis. In addition to this, they also appear to promote vascular leakage *in vitro* and *in vivo* (Esposito et al. 1989; Boyd-White & Williams 1996; Zhang et al. 2015; Wu et al. 2014). There is evidence that AGEs can induce permeability through multiple mechanisms, such as modification of the basement membrane (Boyd-White & Williams 1996) and increased paracellular permeability through Src dependent VE-cadherin phosphorylation (Zhang et al. 2015). Inhibition of Src has also been shown

to attenuate diabetes-induced retinal vascular permeability and VE-cadherin internalisation in the diabetic rat (Kim & Suh 2017). Studies with radiolabelled AGE-BSA have shown that AGE receptors are localised to caveolin-rich membrane domains (Stitt et al. 2000). Caveolae are associated with increased transcellular permeability in the vascular endothelium and are involved with the organisation of receptors to distinct regions at the plasma membrane and intracellularly within vesicles (Bastiani & Parton 2010). Since AGEs induce the upregulation of RAGE (Stirban et al. 2014), could AGE-mediated RAGE upregulation lead to an increase in the number of caveolae? Since caveolae are also involved in mediating transcellular vascular permeability (Guo et al. 2016), AGE/RAGE signalling may therefore increase the number of caveolae, which has been shown to mediate Vegf induced permeability in the retina (Hofman et al. 2000).

Plvap is a marker associated with increased vascular permeability and is expressed on caveolae, and its expression is linked with increased vascular permeability in pathological conditions (Guo et al. 2016). The effect of AGE-BSA treatment on Plvap was analysed by immunofluorescence and qPCR, however, Plvap appeared to be constitutively expressed with no changes observed in either condition. Previous reports that AGE-BSA stimulated an increase in vascular permeability were conducted on endothelial cells grown as a monolayer (Esposito et al. 1989; X.-Y. Zhao et al. 2015). It is possible that the endothelial cells in the metatarsal assay are not sufficiently polarised, which may influence how they respond to permeability-inducing agents. Therefore further investigation *in vitro* of the permeability inducing effects of AGE-BSA should be considered using endothelial cells grown in a

monolayer. Further work could also be performed to ask whether AGE-BSA induces Src activation and disruption of VE-cadherin in this assay.

Since AGE-BSA is known to activate JAK/STAT signalling downstream of RAGE (Stirban et al. 2014) and was shown to induce Il-6 in this assay, which has previously been shown to mediate pathological angiogenesis (Gopinathan et al. 2015), I felt this warranted further investigation. To see if the increase in Il-6 was accompanied by an increase in JAK/STAT signalling, which induces the transcription of pro-inflammatory and pro-angiogenic genes (Scheller et al. 2011), pSTAT3 levels were analysed by IF, and appeared to be increased in AGE-BSA treated metatarsals. However, this was the result of only one independent experiment and the lack of nuclear-specific pSTAT3 staining raises questions about the specificity of the antibody. Nevertheless, it is possible that AGE-BSA directly increases JAK/STAT signalling via RAGE dependent signalling (Xie et al. 2013), which may also be enhanced by the induction of Il-6 (Scheller et al. 2011) through other signalling pathways.

The data presented in this thesis, in agreement with the literature implies that hyperglycaemia mediates a dysfunctional phenotype in endothelial cells associated with decreased Vegf production and angiogenesis, which may be associated with a reduction in pro-survival signalling in endothelial cells (Yu et al. 2016; Busik et al. 2008; Doronzo et al. 2012). In contrast to this, treatment with AGE-BSA is pro-angiogenic, increases Vegf production and appears to bear similarities with angiogenesis in the context of inflammation (Costa et al. 2007). Neither high glucose treatment nor AGE-BSA alone

would be expected to recreate all the effects of diabetes on vascular function that occur in the retina, although a combined treatment of high glucose and AGE-BSA may better represent diabetes-induced vascular changes in the metatarsal model, since the studies in the metatarsal assay have demonstrated that both treatments have very different effects. Despite this, such models have value in that they permit the molecular dissection of the contributions made by individual components of the diabetic phenotype.

One drawback of these studies in the metatarsal assay is that the same dose and treatment regimen was used. Whilst the concentrations used in these experiments follow what has been previously published in the literature and has demonstrated to be effective in eliciting a physiological response in various *in vitro* assays, neither AGE-BSA or high glucose have been administered to the metatarsal assay or other truly similar model before. Therefore some experiments to examine a dose response to both AGE-BSA and high glucose would be useful to find the optimal concentration of both treatments. For example, whilst AGE-BSA is often used at a concentration of 100-500 μg/mL, the concentration of glycated albumin in patients with type 1 diabetes reaches 30 mg/mL (Nathan et al. 2011). Therefore there is a rationale for increasing the concentration of this treatment further whilst still being physiologically relevant. Additionally, if changes to gene expression and angiogenesis occur in a dose dependent manner then it confirms that this response is specific to the treatment.

Another variable to investigate would be the response with time, for both treatments it would be useful to know whether the phenotype increases in severity the longer the cultures are treated for and whether the response changes. For example, there may be a different acute response after the first few days of treatment with either high glucose or AGE-BSA, which could change towards the end of the experiment. In addition to investigating the response over time, investigating whether the phenotype in response to either treatment is reversed when it is removed would hold important functional relevance. In diabetic patients, blood glucose levels are not uniform and fluctuate throughout the day, there is also evidence that high glucose can exert long term epigenetic changes on endothelial cells even after a return to normal levels of glycaemia (Hammes et al. 2011). As a result it would be worth investigating whether high glucose or AGE-BSA can mediate long term changes to the vasculature in this model.

3 Analysis of the diabetic mouse retina

The general consensus presented here in this thesis and in the literature is that *in vitro* and *ex vivo* approaches are useful for studying the vasculature, and play a role in the investigation of some diabetes-induced changes to the vasculature. However, they are unable to fully recreate the complex nature of the vasculature in living tissue. Rodent models of diabetes such as the streptozotocin induced rat and mouse are widely used to investigate diabetes-induced changes to the retina (Lai & Lo 2013). Whilst the streptozotocin rat shows a more severe retinal phenotype in a shorter timeframe after the induction of diabetes, the major drawback of this animal model is that it limits the use of various transgenic models that are primarily available in the mouse. Genetic models of diabetes that exist in the mouse include the $Ins2^{Akita}$ (Barber et al. 2005) db/db (Bogdanov et al. 2014) and Akimba (Wisniewska-Kruk et al. 2014) mouse, all of which show more extensive diabetes-induced changes to the retina than the streptozotocin mouse, and in particular more pronounced change to the retinal vasculature. Whilst the Akimba mouse model exhibits extensive vascular changes such as severe leakage and neovascularisation, the relevance of this model is questionable since it involves the overexpression of Vegf in the photoreceptors (Wisniewska-Kruk et al. 2014). Both the db/db and $Ins2^{Akita}$ mice show evidence of increased vascular permeability, however, the db/db mouse has been reported to show more neurodegenerative features associated with diabetic retinopathy (Bogdanov et al. 2014), which were not observed in the $Ins2^{Akita}$ mouse (Barber et al. 2005). However, microglial activation, reduced astrocyte interaction with the vasculature and altered occludin distribution have been reported in the

Ins2^{Akita} mouse (Barber et al. 2005) which have not been reported in the db/db mouse.

Although the streptozotocin mouse model of diabetes has a less severe retinal phenotype than the db/db and Ins2^{Akita} mouse models, the ability to readily induce diabetes in existing transgenic mouse models is a useful tool for examining the roles of different genes in the pathogenesis of diabetic retinopathy. Additionally, since one of the aims of this thesis was to investigate early diabetes-induced changes to the retina, the streptozotocin-induced mouse is ideal for studying minor changes to the retina.

Analysis of the diabetic mouse retina by *in vivo* imaging and immunohistochemistry revealed, as expected, modest changes to the diabetic retina, consistent with previous reports of minor diabetes-induced changes to the retina in this model (Feit-Leichman et al. 2005; Martin et al. 2004; Lai & Lo 2013). Examination by fundus imaging showed a subtle retinal phenotype with some hyper-reflective spots, which are generally taken to indicate the presence of inflammatory infiltrates, with no changes detected by fluorescein angiography. The lack of observable changes to the vasculature by fluorescein angiography analysis is not surprising after just two months of diabetes, so more detailed analysis was performed on tissue sections and retinal flatmounts. These investigations revealed vascular leakage in diabetic retinas, as judged by staining for endogenous IgG, but not for intravenously administered fluorescein or FITC-dextran. This was unexpected, considering the differences in size between fluorescein (1 kDa),

dextran (40 kDa) and IgG (155 kDa), and given that the lower molecular weight reporters should readily leak from any compromised vasculature.

There are two likely reasons for the disparity in the data from the different methods used. Firstly, imaging by fluorescein angiography (FA) was only recorded up to 10 minutes after the subcutaneous administration of fluorescein, although short time points are unavoidable because of the rapid clearance of fluorescein from the circulation. Other studies have used similar time-points for the analysis of vascular leakage in the laser-induced choroidal neovascularisation (CNV) model of AMD (Takeda et al. 2009). Since the CNV model produces a small focused area of intense leakage and neovascularisation and is thus well-suited to FA, this method and time-point may not be sensitive enough to detect the more modest levels of vascular leakage in the diabetic retina. This may also explain why FITC-dextran perfusion (1 hour) similarly failed to reveal any areas of leakage, as one hour may not have been long enough to allow sufficient extravasation of the fluorescent tracer.

A second possible explanation is that since the conditions in the retina are likely to be dynamic and constantly changing, the permeability of the retinal vasculature may also vary with natural fluctuations in blood glucose levels and diurnal alterations in vascular permeability possibly due to dark-induced stress on the retina (Arden et al. 1998). Whilst any endogenous extravasated IgG is likely to be slowly broken down and cleared from the retina, measuring the level of an endogenous marker of vascular leakage permits analysis over a longer time period than a snapshot obtained with

exogenous tracers, and will detect episodes and sites of transient vascular permeability. Sacrificing mice at other times during the day such as one hour after light onset or during darkness may also reveal a retinal phenotype with increased vascular leakage. However, it is not clear how specific this predicted increase in vascular leakage would be to the diabetic retina.

Aside from vascular leakage, other markers of altered retinal phenotype were analysed. GFAP is constitutively expressed in astrocytes and expressed in Müller cells during periods of retinal stress (de Hoz et al. 2016). However, staining retinal sections revealed only minor changes in GFAP expression in diabetic animals. It has been reported that GFAP expression is increased in Müller cells in the diabetic rat (Ly et al. 2011) and the db/db diabetic mouse (Bogdanov *et al.*, 2014), and whilst there is one report of increased GFAP in the streptozotocin mouse retina (Feit-Leichman et al. 2005), this was analysed by western blot, which does not indicate in what cell type the increase occurred, and was transient as GFAP returned to basal levels after the initial increase at one month post diabetes induction. Based on the lack of convincing changes alterations to GFAP in the retina of the streptozotocin mouse, it is possible that increased GFAP expression in the Müller cell does not occur in this model at this early stage.

Since analysis of the sectioned diabetic mouse retina revealed little evidence of GFAP expression in Müller cells, retinal flatmounts were analysed for changes in astrocytes and their interaction with the retinal vasculature. In contrast to observations reported in the diabetic rat retina

(Ly et al. 2011; Barber et al. 2000) and Ins2^{Akita} mouse (Barber et al. 2005), no significant or observable changes in the numbers of astrocytes or their interaction with the vasculature were observed. The lack of any visible changes in the biology of the retinal macroglia is surprising, as neurovascular coupling plays a key role in maintaining the blood-retinal barrier (Antonetti et al. 2006), and since endogenous IgG staining revealed clear evidence of vascular leakage, it was surprising to find no disturbance of altered glial-endothelial cell interactions. The observations here in the mouse are in contrast with data from the streptozotocin rat and Ins2^{Akita} mouse that revealed a decrease in GFAP in the astrocytes (Barber et al. 2005; Ly et al. 2011; Barber et al. 2000). One thing this study may imply is that changes to the retinal macroglia are transient, and only occur during the very early stages of diabetes and rapidly resolve.

Whilst changes to the vasculature are important indicators of the health of the retina in diabetes, other changes may precede more noticeable alterations to the vasculature, and of particular importance are those that are associated with and promote inflammation. Microglia are resident immune cells in the retina and CNS, and become activated in response to inflammatory stimuli (Tang & Kern 2011). Inhibition of microglial activation has been demonstrated to reduce inflammation in the diabetic rat retina (Krady et al. 2005), which suggests they play an important role in mediating diabetes-induced inflammation in the retina. In diabetic mouse retinas, Iba1 expressing cells, which are likely to either be resident microglia or blood derived macrophages, had adopted an amoeboid structure and appeared to be migrating into the inner nuclear and ganglion

cell layers. This change in Iba1^{+} cell morphology has also been described in the Ins2^{Akita} mouse (Barber et al. 2005), however, since this was analysed in retinal flatmounts, it does not so readily show the migration between different layer of the retina. Migrating microglia/macrophages have also been shown to migrate to the ganglion cell and outer plexiform layers of the rat retina in diabetes (Stitt et al. 2016), in agreement with data in this thesis.

Oxidative stress is considered to be a hallmark feature of diabetes-induced vascular dysfunction (Giacco & Brownlee 2010), and has long been acknowledged to mediate the pathophysiology of diabetic retinopathy (Kowluru & Chan 2007). Since oxidative stress plays such an important role in diabetic retinopathy I decided to investigate this by immunofluorescence analysis of 4-HNE, a marker of lipid peroxidation. Staining for 4-HNE revealed an increase in immunoreactivity in diabetic retinal sections that was particularly evident in the photoreceptor layer. This is in agreement with a previous report that showed photoreceptors contribute to diabetes-induced oxidative stress (Du et al. 2013). It is also unsurprising to see the greatest amount of oxidative stress in photoreceptors, since they have the highest metabolic activity in the retina (Kern & Berkowitz 2015) and are abundant in mitochondria which are a major producer of superoxide, a strong oxidising agent (Kanwar et al. 2007). Additionally, photoreceptors outer segments are formed of stacks of folded membrane which contains the light-sensing GPCR rhodopsin (Kocaoglu et al. 2016). The membrane-rich nature of the photoreceptor means there is an abundance of lipids, which can become peroxidated.

Activation of the Nf-κb signalling pathway is a common feature of inflammation and drives the expression of classic pro-inflammatory genes such as Il-1β, Il-6 and Tnf-α (Lawrence 2009). To examine inflammatory changes in the diabetic retina, and localise these changes within the retina, analysis of Nf-κb activation was performed. Although no evidence of Nf-κb signalling was revealed by immunofluorescence analysis of phospho-p65, western blotting indeed revealed an increase in Nf-κb phosphorylation in the diabetic retina. In agreement with data in this thesis, an increase in Nf-κb phosphorylation by western blot analysis has also been reported by others in the streptozotocin mouse two months after diabetes induction (Wang et al. 2010). Whilst the results clearly differ between the two methods of analysis in this thesis, this was most likely due to the antibody exhibiting greater affinity and specificity for an epitope(s) in the denatured protein. In addition, it is possible that the antibody failed to adequately penetrate through to the nucleus in the retinal sections to permit detection of the p65 subunit. Nevertheless, both immunofluorescence analysis of Iba1 staining and western blotting of Nf-κb provided evidence of a pro-inflammatory state in the diabetic retina which is consistent with the involvement of both Nf-κb signalling and activated microglia in the pathophysiology of diabetic retinopathy prior to the development of vascular leakage. The data presented in this thesis and by others (Wang et al. 2010; Joussen et al. 2001; Du et al. 2013) show that inflammation is a key first step in the early stages of diabetic retinopathy, and therefore may be an effective therapeutic target for the early stages of diabetic retinopathy.

4 Altered gene expression in the diabetic mouse retina

To identify factors that could potentially mediate diabetes-induced changes to the retina, qPCR analysis was used to examine changes in gene expression. As expected, *Vegf* expression was increased in the diabetic retina, consistent with its well established and central role in promoting vascular leakage (Salam et al. 2011; Bai et al. 2009; Wang et al. 2010; Simó et al. 2014). Of greater interest here, given the objective of identifying novel candidate therapeutic targets, other factors such as *Angptl2*, *Angptl6*, *Lrg1* and *Tgfβ* were also upregulated in the diabetic retina. As previously discussed all these factors have effects on the vasculature, and may play a role in the pathophysiology of diabetic retinopathy. It is also interesting to note differences in the standard distribution of data from control and diabetic mice. The increase in standard deviation in diabetic mice compared to controls shows that despite the same genetic background, altered gene expression in diabetes varies significantly between individual animals. This may in turn correlate with a wide variation in the extent of any retinal pathology, and is not dissimilar to the situation in patients with diabetes where there is a broad spectrum of retinal disease.

4.1 Angptl6

The increase in expression of several genes during this early stage of diabetes in the mouse retina shows that diabetes induces altered gene expression prior to the onset of any physical changes to the retina. Further work to establish the exact roles of these upregulated genes in the diabetic retina would improve our understanding of the early stages of the pathophysiology of diabetic retinopathy. In the first instance, I decided to explore the role of Angptl6 on the vasculature in more detail, since little is known about its effect on the vasculature, apart from the fact it induces endothelial cell migration and vascular permeability (Oike et al. 2004; Okazaki et al. 2012). In order to do this, I utilised the mouse metatarsal assay, due to the merits of this assay which have been discussed earlier in this chapter and elsewhere (Deckers et al. 2001; Song et al. 2015).

However, ANGPTL6 did not increase angiogenesis in this assay, unlike VEGF, which was used as a positive control. Whilst this finding contradicts previously published reports demonstrating ANGPTL6 to be pro-angiogenic both *in vitro* and *in vivo* (Oike et al. 2004), there may be several reasons for the absence of a response in the metatarsal assay. First of all, the authors who initially reported the pro-angiogenic effects of Angptl6 produced the recombinant mouse protein in house, whilst only the human recombinant protein is commercially available. Whilst mouse and human recombinant proteins are frequently used interchangeably, it is plausible that the recombinant human protein has reduced activity on mouse cells. The receptors specific for the Angptl growth factors have not been as well characterised as those for the angiopoietins, but Angptl6 has been shown to

signal through certain RGD-containing integrins, this RGD motif is conserved in the human and rodent orthologues (Zhang et al. 2006). Conservation of this motif implies that binding to these integrins is critical to the biology of this growth factor. The expression of different integrins has not been investigated in the metatarsal assay, however, two RGD binding integrins $\alpha_5\beta_1$ (Kim et al. 2000) and $\alpha_V\beta_3$ (Brooks et al. 1994) have been shown to be essential for angiogenesis *in vivo* so would be expected to be expressed in the metatarsal assay. Additionally, half of all known integrins bind to the RGD motif (Ruoslahti 1996), so it is likely that the metatarsal expresses at least some integrins capable of recognising ANGPTL6, which means it is therefore surprising that this growth factor did not induce angiogenesis. However, it is important to note that by culturing the embryonic bones on a gelatin-coated tissue culture plate, this may artificially alter the expression of integrins on endothelial cells compared to what is found in other conditions and/or *in vivo*, which may in turn impair the ability of endothelial cells to respond to ANGPTL6.

4.2 Lrg1

Whilst *Angptl6* expression was increased in the diabetic mouse retina, further work to elucidate its functional role had limited success. Experiments in the metatarsal assay failed to demonstrate its ability to induce angiogenesis, and experiments in cultured endothelial cells (HUVEC and primary mouse brain derived endothelial cells) failed to identify any striking changes to cell signalling associated with angiogenesis (experiments not shown in this thesis). Whilst further work could have been pursued, such as using inhibitors or knock out mice to investigate the effect of inhibiting the endogenously produced protein or the sourcing of the recombinant protein from another source to confirm its efficacy, other lines of research were pursued instead.

Whilst LRG1 has been described as a pro-angiogenic and vascular permeability inducing factor which is increased in the vitreous of patients with proliferative diabetic retinopathy (Wang et al. 2013), its potential role in mediating early changes in the pathophysiology of diabetic retinopathy has not been explored. Since other members of the lab have researched the role of this protein in mouse models of Age-related macular degeneration (AMD), another leading cause of blindness similar, its role in diabetic retinopathy was of relevant interest. Additionally since the recombinant protein, blocking antibody and knockout mouse are readily available to use this allows a greater number of functional experiments to be readily performed.

LRG1 exerts its effect by activating the TGF-β angiogenic switch, whereby it alters the conformation of the TGF-β receptor complex from TGFβR2/ALK5 to TGFβR2/ALK1/Endoglin. The change in conformation of the receptor complex alters downstream signalling from Smads 2 and 3, which promote vascular quiescence, to Smads 1, 5, and 8, which is pro-angiogenic. Due to context of TGFβ signalling (Pardali et al. 2010), wholesale blockade of TGFβ signalling would be inappropriate. In fact, deletion of *Tgfβr2* in the eye induced severe vascular defects characteristic of diabetic retinopathy (Braunger et al. 2015), implying that TGFβ signalling plays a role in maintaining the integrity of the retinal vasculature. Therefore, targeting LRG1, which is specifically involved in mediating pro-angiogenic TGFβ signalling, is less likely to have deleterious effects, in contrast to TGFß or VEGF blockade.

Having shown that *Lrg1* is upregulated along with *Tgfbeta* in the diabetic mouse retina, the role of Lrg1 warranted further investigation. Fundus imaging was initially used to allow the retinas of diabetic Lrg1 KO and WT mice to be examined non-invasively. As is characteristic of this model, the retinas exhibited patchy bright spots typical of inflammatory infiltrates, though this phenotype appeared to be less severe in the Lrg1 KO mice. To determine whether diabetic Lrg1 KO mice had reduced vascular leakage compared to WT mice, flatmounted retinas were stained for endogenous IgG. Imaging of the retinas revealed that there were fewer focal regions of vascular leakage in diabetic Lrg1 KO mice compared to diabetic WT. These observations are consistent with the known function of Lrg1 as a pro-angiogenic, pro-permeability factor (Wang et al., 2013), and suggest that

loss or inhibition of Lrg1 may be protective against diabetes-induced vascular leakage in the mouse retina. In future studies it would be interesting to see if Lrg1 KO mice also had reduced Nf-κb activation, microglial activation or reduced oxidative stress, and whether in the diabetic Lrg1 KO mice there were any changes in the induction of Vegf and Angptl2 and 6. If so, this would support the idea that Lrg1 plays a role in the pathophysiology of diabetic retinopathy prior to the onset of vascular dysfunction, and suggests that LRG1 blockade in patients prior to the onset of vascular symptoms may limit progression of the disease.

Since Lrg1 KO mice presented a less severe retinal phenotype in diabetes, I sought to investigate the effects of diabetes on the retinas of human LRG1 knock-in (KI) mice. A lack of suitable reagents had prevented me from confirming at the protein level that Lrg1 was increased in the diabetic retina in WT mice, but the hLRG1 KI mice might allow analysis of LRG1 protein expression through a sensitive ELISA developed in the lab. However, the concentration of LRG1 in the retinal lysates was below the detection limit of the ELISA (data not shown). A second aim of the studies with the hLRG1 KI mice was to confirm that these mice responded in the same way as WT mice to diabetes induction. Replacement of the mouse gene with the human cDNA, albeit at the correct locus in the mouse genome, could potentially lead to altered regulation of transcription and translation compared to the endogenous mouse gene. This could be mediated by differences in the affinity of transcription factors to promoter sequences within the coding region of this gene, due to slight differences in the sequence of human and mouse LRG1. In addition, since the cDNA sequence was inserted, it may

lack non-coding RNA enhancer sequences present in the intron (Li et al. 2016), an example of which is found in the intronic sequence of IL-4 (Hural et al. 2000).

Therefore it was important to ensure that these mice had a diabetic retinal phenotype similar to WT mice to validate this mouse model before further experiments were conducted. Analysis of endogenous IgG by immunofluorescence indeed confirmed that a similar retinal phenotype developed after diabetes induction as in WT mice. This finding indicates that this model is suitable for further study into the role of Lrg1 in the diabetic retina, and may be particularly useful for *in vivo* testing of a humanised function-blocking anti-LRG1 antibody developed in the lab.

In summary, data in this thesis from the investigation of the diabetic retina highlights an important role for inflammation in mediating early diabetes-induced pathophysiological changes as described by the increase in Nf-κb phosphorylation, altered microglial/macrophage activation and migration, and oxidative stress, which are in agreement with data from the streptozotocin mouse and other models of diabetic retinopathy. Whilst neither high glucose nor AGE-BSA treatment in the metatarsal assay completely recreated diabetes induced vascular dysfunction in the metatarsal assay, the pro-angiogenic and pro-inflammatory effect of AGE-BSA treatment means that it has greater potential for investigating inflammation-induced vascular dysfunction. Further work to explore the mechanisms behind diabetes-induced retinal inflammation could therefore identify new therapeutic targets, which could be utilised in the clinic prior to

the onset of vascular dysfunction. More specifically, characterising the role of Lrg1 in diabetes-associated vascular changes may indicate a potential utility for LRG1 blockade in the early stages of diabetic retinopathy.

This research in the diabetic mouse retina has identified three candidate factors with potential for use in the management of diabetic retinopathy. Whilst LRG1 cannot be considered as a new therapeutic target, the research in this thesis can be considered has acted as a 'proof of concept' that LRG1 is involved in the pathogenesis of diabetic retinopathy and existing therapeutics which are currently being developed could be utilised to treat diabetic retinopathy.

Two other factors were identified, Angptl2 and Angptl6 in the diabetic mouse retina. Unlike Lrg1, which has already been characterised and established as an appropriate therapeutic target, Angptl2 and Angptl6 have not been as well characterised. Further work must be done before either candidate can be considered to be an effective therapeutic target. In order to do this, one must demonstrate that Angptl2 and/or Angptl6 mediate an aspect(s) of the pathophysiology of diabetic retinopathy, which can be effectively targeted without having a deleterious off-target effect. Research into both factors could be performed to see if the absence of either has a protective effect on the diabetic retina without causing deleterious effects on the retina. Additionally administration of either factor to the retina in non-diabetic mice should be able to recreate some of the features of diabetic retinopathy in these mice, this would confirm that this factor contributes to specific features of diabetic retinopathy. Secondly, these

factors should also be confirmed to be increased in human patients with diabetic retinopathy, ethical approval would have to be sought to utilise human vitreous samples to test this hypothesis in humans. Having confirmed this, an effective mechanism of blocking or inhibiting the effect of either factor should be designed which acts specifically on either factor without any adverse effect. If the described studies above demonstrate a clear role for either Angptl2 or Angptl6 this would generate data sufficient for publication in a peer-reviewed journal with a strong translational aspect which could lead to the development of a novel therapeutic.

In addition to the work to perform with the aim of identifying novel therapeutic targets described above, further studies with the metatarsal assay could be performed with the aim to identify mechanisms leading to diabetes-induced vascular regression. Whilst hyperglycaemia did not alter pericyte coverage in this model, the behaviour of other cell types such as smooth muscle cells and fibroblasts may instead be altered which could potentially lead to impaired tissue vascularisation. I believe that these other cell types may be reduced or otherwise negatively affected by hyperglycaemia, which would impair endothelial cell growth and survival.

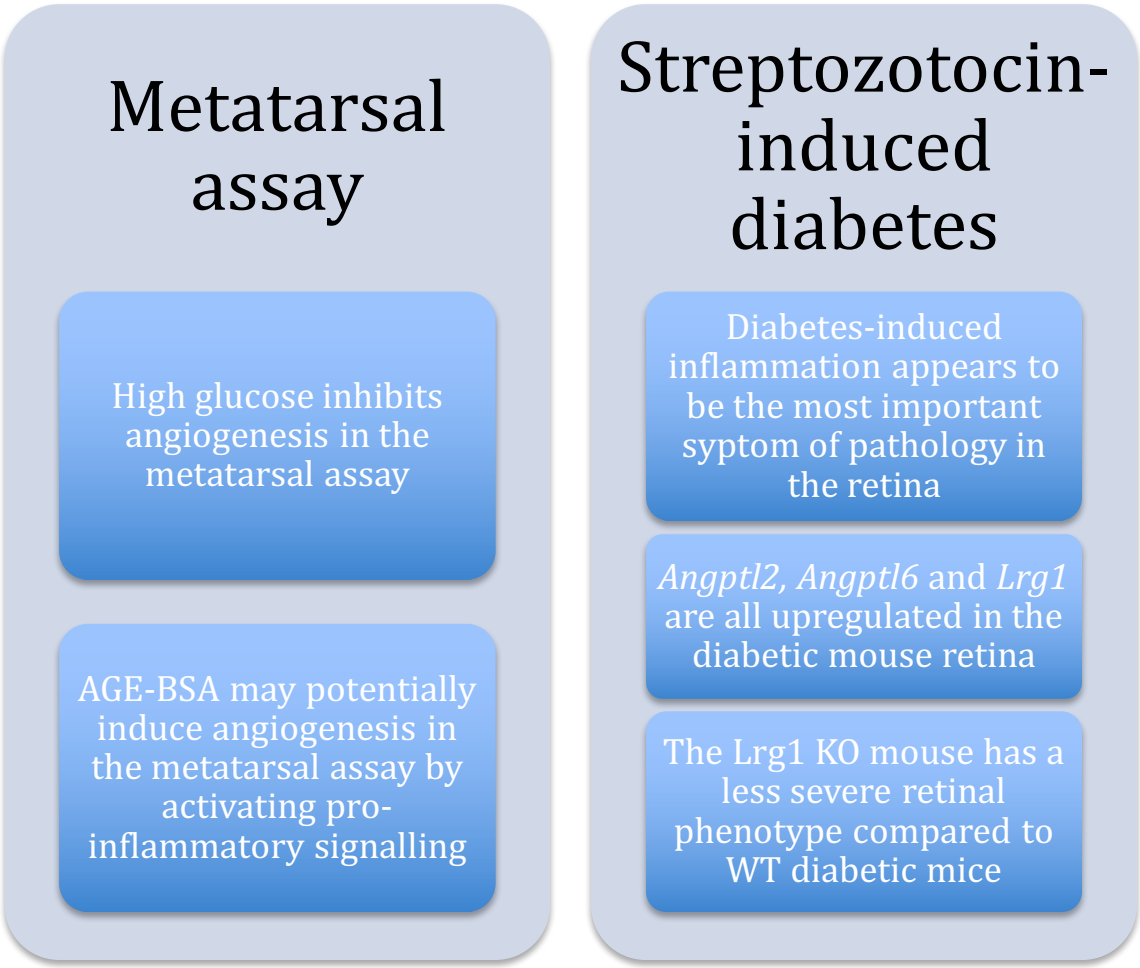

Figure 31: A summary of the main findings of this thesis.

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